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## **Development and validation of a UHPLC-ESI-QTOF mass spectrometry method to analyze opines, plant biomarkers of crown gall or hairy root diseases.**

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## **Abstract**

Opines are low-molecular-weight metabolites specifically biosynthesized by agrobacteria-transformed plant cells when plants are struck by crown gall and hairy root diseases, which cause uncontrolled tissue overgrowth. Transferred DNA is sustainably incorporated into the genomes of the transformed plant cells, so that opines constitute a persistent biomarker of plant infection by pathogenic agrobacteria and can be targeted for crown gall/hairy root disease diagnosis. We developed a general, rapid, specific and sensitive analytical method for overall opine detection using ultra-high-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-MS-QTOF), with easy preparation of samples. Based on MS, MS/MS and chromatography data, the detection selectivity of a wide range of standard opines was validated in pure solution and in different plant extracts. The method was successfully used to detect different structural types of opines, including opines for which standard compounds are unavailable, in tumors or hairy roots induced by pathogenic strains. As the method can detect a wide range of opines in a single run, it represents a powerful tool for plant gall analysis and crown gall/hairy root disease diagnosis. Using an appropriate dilution of plant extract and a matrix-based calibration curve, the quantification ability of the method was validated for three opines belonging to different families (nopaline, octopine, mannopine), which were accurately quantified in plant tissue extracts.

## **Keywords**

Opines, detection, quantification, UHPLC-ESI-MS-QTOF, diagnosis, agrobacterium

## 1. Introduction

Crown gall and hairy root are plant neoplastic diseases inducing uncontrolled overgrowth of plant tissue (tumors) or anarchically proliferating roots, respectively. These diseases can affect nursery and mature plants, and are among the most detrimental ones for fruit trees and ornamental plants from an economic point of view [1–3]. Economic losses ensue not only from intrinsic damage to infected plants, but also from their prohibited commercial use [3,4]. Both diseases are caused by agrobacteria, common soil-borne Alphaproteobacteria belonging to the *Rhizobiaceae* family [5]. They usually live saprophytically in many plant rhizospheres, but occasionally adopt a phytopathogenic lifestyle and infect wounded plants, namely most of dicotyledonous species, and a few monocots and gymnosperms [6]. This occasional virulence is determined by the presence of a large (at least 200 kb) DNA plasmid: the tumor-inducing (Ti) and root-inducing (Ri) plasmids provoke crown gall and hairy root diseases, respectively [7]. These plasmids often occur in bacteria of the genus *Agrobacterium*, but they are also harbored by other genera of *Rhizobiaceae*, e.g., *Allorhizobium*, *Rhizobium*, *Pararhizobium* or *Neorhizobium* [8]. Briefly, the pathogenicity of the Ti and Ri plasmids involves virulence genes mainly located on these plasmids that mediate the transfer of T-DNA (a small portion of these plasmids, ca. 20 kb) to the nuclear DNA of the host plant cells where it is incorporated into the plant genome. T-DNA genes are thus expressed in these transformed plant cells [9–11]. Besides causing uncontrolled cell division (a crown gall tumor or hairy roots), the T-DNA encodes information for the biosynthesis of unusual low-molecular-weight metabolites only produced by the transformed plant cells, referred to as opines [12].

More than 30 crown gall opines have been characterized to date [13–15]. The first opine described in the literature is octopine, isolated from a muscle extract of *Octopus octopodia* [16]. Five opines have also been identified to be important end-products of the anaerobic metabolism in a range of marine invertebrates [17–19]. Opines are a heterogeneous group of molecules divided into two structure classes: (i) sugar phosphodiester, called agrocinopines, and (ii) amine derivatives resulting from the condensation of one amino acid with an alpha-ketoacid or a sugar, which include most of opines [12,20]. This latter chemical class is subdivided into 8 opine families, depending on their composition and their biosynthesis pathways; the most frequently encountered ones are the nopaline, octopine, and mannityl-opine families [21]. For a review of opine families and structures see Dessaux *et al.* [13,15]. Among the several types of Ti and Ri plasmids, each one allows for the biosynthesis and catabolism of a few of these compounds. Ti or Ri plasmid-harboring agrobacteria use opines as specific carbon, nitrogen or sulfur sources, whereas most other microorganisms are unable to use them [22,23]. Furthermore, some opines induce the conjugative transfer of these plasmids to neighboring non-pathogenic agrobacteria and thus promote the dissemination of pathogenicity [24–27].

No curative method is available for these plant diseases. Early diagnosis followed by sanitation procedures like uprooting can prevent pathogenic agrobacteria from disseminating and settling in crops [3], but this requires a rapid, sensitive and specific method to detect this bacterial infection in plants. As T-DNAs are sustainably incorporated into the genomes of the transformed plant cells, the presence of opines in plant tissue constitutes a persistent biomarker of plant infection by a pathogenic agrobacterium targetable for crown gall/hairy root disease diagnosis. For example, an opine-based biosensor has recently been proposed to specifically detect nopaline and octopine, and tell whether tumors are induced by *Agrobacterium* or not [28]. Nevertheless, opines are not all biosynthesized concomitantly in abnormal tissue outgrowths because their production depends on the type of plasmid harbored by the inciting agrobacterium. According to the plasmid, diverse types of opine biosynthesis genes are transferred, and generally more than one [29]. Opines produced in transformed plants also seem to be dependent on the precursors available in the plant [14]. Therefore, the development of a general method to detect various opines in plant extracts is needed to use opine-based diagnostic techniques for these plant diseases.

Different analytical techniques and methods have been developed and used to detect and analyze opines, such as enzymatic assay [30,31], paper/thin layer chromatography (TLC) [32] or high-voltage paper

electrophoresis (HVPE) followed by chemical staining [29,33–36], high-performance liquid chromatography (HPLC) with diverse detectors (fluorescence, UV absorbance at 254 nm, conductivity detection, refractometry) associated or not to a previous step of opine-type-specific derivatization [17,34,37–39], gas chromatography coupled to mass spectrometry (GC-MS) with previous specific derivatization [40], or liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) with previous butylation [18]. Each of these methods has unique features, so that only a few types of opines can be studied. Moreover, some can present drawbacks: TLC and HPVE have limited sensitivity (high detection limits) and do not provide accurate quantification [34], whereas GC-MS and HPLC coupled to various detectors are more sensitive and allow for a quantification procedure. However, they all present main disadvantages such as (i) partial purification pretreatment of the plant or mollusk extracts before analysis by GC-MS [40] or HPLC-UV [37], and/or (ii) the use of reagents to reveal the compounds [29,32–36], or (iii) additional steps of derivatization for analysis by GC-MS [40] or for fluorescence or UV detection [34,37,39]. All these steps not only imply a time-consuming process before analysis, but also fail to detect all opine types in one go because they require different chemical staining procedures (cited in [34]) or derivatizations according to the targeted opine structures [17,34,37–39]. Furthermore, natural extracts are often complex mixtures and could contain other compounds (such as amino acids) also revealed by the reagents or the derivatization procedures. The detected compounds have to be compared with opine standards (not always available) to be unequivocally identified as opines, especially when UV or fluorescence detectors are used [34,37,39]. HRMS provides additional chemical information (accurate mass, chemical formula, fragmentation pattern ...) to further identify a compound within a complex extract [18].

In the present study, an analytical method was developed for overall opine detection based on ultra-high-performance liquid chromatography–electrospray ionization quadrupole time-of-flight–mass spectrometry (UHPLC-ESI-MS-QTOF), with easy preparation of non-derivatized samples. This method proved useful not only to detect but also to quantify different types of opines in plant extracts. The quantification method was validated for opines belonging to three different families, *i.e.*, nopaline-, octopine-, and mannityl-opines.

## 2. Material and Methods

### 2.1. Chemicals and reagents

Nopaline and octopinic acid were purchased from Sigma-Aldrich Laboratory (Saint Louis, Missouri, USA), and octopine from ICN Biomedicals Inc. (Aurora, Ohio, USA). Mannopine, mannopinic acid, agropine, agropinic acid, cucumopine, histopine, alanopine, pyronopaline, agrocinopine A, and some synthetic non-natural opine analogs like allo-octopine (a diastereoisomer of octopine), galactopine and glucopine (isomers of mannopine), galactopinic and glucopinic acids (isomers of mannopinic acid) and nor-mannopine (a synthetic opine), were kindly supplied by Y. Dessaux, D. Faure and S. Morera (Institute for Integrative Biology of the Cell, Gif-sur-Yvette, France). The chemical reagents and solvents such as acetonitrile, water and formic acid (UHPLC-MS grade) were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA).

### 2.2. Bacterial strains and plant materials

The bacteria used in this study are listed in Table S1 (see Electronic Supplementary Material, ESM1). Plant experiments were conducted on the following plant species grown in a greenhouse: *Solanum lycopersicum* (tomato), *Rosa* sp., *Kalanchoe daigremontiana*, and *in vitro* cultures of wild-type and transgenic *Lotus corniculatus* (bird's-foot trefoil) modified to produce nopaline and mannopine [41,42].

### 2.3. Inoculation, culture, harvesting and extraction

Prior to plant infection, bacterial strains were grown overnight under shaking (160 rpm) at 28°C in YPG-rich medium (yeast extract 5 g/L; peptone 10 g/L; glucose 10 g/L; pH 7.2). The bacteria were then washed and

suspended in sterile water at a concentration of  $10^8$  per mL. Ten microliters of the suspensions (*i.e.*  $10^6$  bacteria) were inoculated on the stem collar zone of the plants after incising with a scalpel blade. The plants were then incubated 21 days in a greenhouse with 16 hours illumination, at 22°C (day) and 19°C (night).

Each plant tumor was harvested and immediately frozen in liquid nitrogen (metabolism quenching) and stored at -80°C. Freeze-dried tumors were ground with a benchtop homogenizer (FastPrep-24™, MP Biomedicals™, Fisher Scientific, UK), and 30 mg of powdered samples were subjected to two successive extractions by adding 1 mL of a methanol/H<sub>2</sub>O 20:80 (v/v) solvent mix and 15 min sonication at each extraction step. After centrifugation (10 min, 19745 × g), the supernatant was evaporated to obtain the crude extract. For the *in vitro*-cultivated transgenic *Lotus corniculatus*, the extraction process was applied to entire plants. All these extracts were solubilized at 10 mg/mL in UHPLC-MS grade water and stored at -20°C until analyses.

Blank plant matrices consisted of non-transformed, opine-less plant tissue extracts. They were obtained following the same extraction procedure, but using stem samples of non-inoculated plants (for tomato and kalanchoe) or entire plants for *in vitro*-cultivated wild-type *L. corniculatus*.

## **2.4. Preparation of standard solutions, calibration standards and quality control samples**

Each stock solution of opine standard was prepared at 1 mg/mL in UHPLC-MS grade water and stored at -20°C. Two mixed standard solutions were also prepared. They contained equal quantities of each compound, namely either all 16 tested opines (*Mix16*) or just three of them: nopaline, octopine and mannopine (*Mix3*). These mixed standard solutions were prepared in UHPLC-MS grade water or in blank plant matrix solutions at extract concentrations of 10 mg/mL, 0.5 mg/mL and 0.1 mg/mL. For the linearity study, the *Mix3* standard solutions were also diluted to get concentrations of each opine ranging from 10 to 15000 ng/mL using either UHPLC-MS grade water or the appropriate concentration of blank plant matrix solution. All opines were also spiked separately (1 µg/mL) in the same blank plant matrix samples for quality control (QC).

## **2.5. UHPLC-ESI-MS-QTOF analysis: instruments and analytical conditions**

An Agilent 1290 UHPLC system coupled with an Accurate-Mass Q-TOF 6530 spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used. Liquid chromatography was carried out using a Poroshell 120 EC-C<sub>8</sub> column (2.7 µm particle size, 100 mm x 3 mm, Agilent Technologies, Newport, CA, USA) at 30°C. The mobile phase was a mixture of acetonitrile and acidified water (0.4% formic acid) with a flow rate of 0.6 mL/min. The gradient started with 100% acidified water for 3 min, and then increased linearly to 100% acetonitrile in 4 min, maintained for 1 min before cycling back to the initial conditions, and then kept for post-acquisition column equilibration (2 min). The injection volume of each extract or standard solution was 10 µL. The quadrupole time-of-flight mass spectrometer (QTOF-MS) equipped with an electrospray ionization source (ESI Agilent Jet Stream thermal gradient focusing technology, Santa Clara, CA, USA) was used in positive ionization mode for MS and tandem MS analyses under the following conditions: drying gas (N<sub>2</sub>) flow of 11 L/min at 310°C, nebulizer pressure of 40 psi, sheath gas flow rate of 11 L/min at 350°C, with the capillary, nozzle and fragmentor voltages set to 3000 V, 500 V, and 100 V, respectively. The acquisition mass range was *m/z* 80 to 2000 with a scan rate of 2 spectra/s, and the MS<sup>2</sup> experiment was done with the collision energy set at 10, 20, 30 or 40 V. A reference solution containing a standard compound (HP-0921) at *m/z* 922.00979 was constantly infused as an accurate mass reference. The UHPLC-ESI-MS-QTOF device was managed by Mass Hunter Workstation Acquisition B.07.00 software, and the data was reworked with Mass Hunter Qualitative Analysis B.07.00 software (Agilent Technologies).

## **2.6. Validation of the quantification method**

The quantification method was validated as described in the literature [53–58] on *Mix3*, containing nopaline, octopine and mannopine. The parameters were selectivity, linearity, sensitivity, intra- and inter-assay precision

and accuracy, extraction efficiency (recovery), stability, carry-over, and matrix effect. Selectivity of detection was also verified for all studied opines.

#### **2.6.1. Selectivity (specificity)**

Selectivity refers to the ability of the method to precisely detect and unequivocally distinguish a particular compound from all the other endogenous components present in a complex mixture or in the solvent. For each opine, selectivity was assessed by analyzing UHPLC-grade water samples (the solvent used for sample preparation,  $n=5$ ) and blank plant matrices ( $n=5$  for tomato and kalanchoe;  $n=3$  for bird's-foot trefoil) compared to standard solutions and spiked blank plant matrices (QC). The presence of potential interfering components co-eluting with each opine was investigated based on retention time and accurate mass. MS/MS fragmentations were studied when required. The specificity of the method was validated for each opine when no interfering peak was observed for its characteristic ions at the expected retention times.

#### **2.6.2. Linearity (Calibration curve)**

Linearity was evaluated by analyzing three or four different calibration curves for each opine. Twelve concentration levels were tested: 10, 50, 100, 250, 500, 750, 1000, 2500, 5000, 7500, 10000 and 15000 ng/mL (prepared as described in 2.4). For each opine standard, the slope and the y-intercept of the calibration curve were calculated, as well as the coefficient of determination ( $R^2$ ). The linearity range was established as the interval between the limit of quantification (LOQ) and the highest concentration of the compound which still maintained a good linearity. It was considered satisfactory when the  $R^2$  of the calibration curves was higher than 0.990 for all the curves.

#### **2.6.3. Sensitivity (LOD and LOQ)**

The limit of detection (LOD) for each opine was defined as the lowest detectable concentration and was calculated as  $3\sigma/S$  ( $\sigma$  is the standard deviation of the y-intercepts of the regression lines and  $S$  the slope of the calibration curve) [53]. The LOQ was also defined for each opine as the lowest concentration that could be quantified and calculated as  $10\sigma/S$  [53].

#### **2.6.4. Intra- and inter-assay precision and accuracy**

Accuracy and precision were assessed by analyzing *Mix3* at four concentrations (250, 500, 750 and 1000 ng/mL). Intra-assay precision and accuracy were estimated by repeated measurements ( $n=4$ ) in a single run. Inter-assay precision and accuracy were estimated by injecting the same standard solutions on three different days ( $n=3$ ). The means, standard deviations, relative standard deviations (% RSD) and percent deviations of the calculated concentrations (% bias) were obtained for each standard level. Precision (% RSD) and accuracy (% bias) were considered as acceptable if  $<15\%$  ( $<20\%$  at the LOQ level) [54,56].

#### **2.6.5. Extraction efficiency (Recovery)**

Extraction efficiency was determined by comparing the peak areas obtained from a known amount of each opine (1  $\mu\text{g/mL}$ ) spiked in a blank plant sample before or after extraction ( $n=4$ ). The spiked blank plant sample was dried before extraction. Results were expressed as percentages of recovery between the two signals [54,56].

#### **2.6.6. Matrix effect**

In quantitative LC-MS bioanalysis, matrix effects can cause ion suppression or enhancement because the complex matrix composition can influence the ionization of the targeted compound, providing incorrect data that negatively affect the performance of the study. The matrix effects were assessed by performing the following two experiments: (1) the matrix effect of blank plant matrix (the crude extract at 10 mg/mL) on opine ionization was evaluated by comparing the peak areas of the targeted opine spiked at 500 ng/mL in the blank plant matrix

to the peak areas obtained by injecting the neat standard solution at the same concentration in water; (2) quantifications were performed for a set of four opine concentrations (250, 500, 1000 and 2500 ng/mL) spiked in a 100-fold diluted blank plant matrix (corresponding to 0.1 mg/mL of crude extract). The quantified values were calculated from either a solvent-based calibration curve or a matrix-based calibration curve (with a 100-fold diluted blank plant matrix). The matrix effects on the analytical recovery rates of each opine were evaluated by comparing these quantification results ( $n=3$ ). Recovery rates were expressed as percentages of the expected value and considered as acceptable when  $> 85\%$ . Relative standard deviations (% RSD) were also calculated.

#### 2.6.7. Stability

Three opines at low, medium and high concentrations (250, 750 and 1000 ng/mL) were analyzed at three different dates within a 12-day period to evaluate their stability, with storage at  $-20^{\circ}\text{C}$  and freeze-thaw cycles between each analysis. The analytical signals obtained for each opine concentration were compared between days, and the relative standard deviations (% RSD) were calculated and considered acceptable when  $<15\%$  ( $<20\%$  at the LOQ level) [57].

#### 2.6.8. Carry-over

Carry-over was evaluated by analyzing a solvent blank right after analyzing the highest concentration level of the calibration curve or the sample containing the greatest concentration of the targeted opine. The extracted ion chromatograms (EIC) of the targeted opine were studied to track residues from the first injection in the second injection. If a carry-over phenomenon was detected, it was considered as acceptable for quantification when below 20% of the LOQ [56,58].

### 2.7. Statistical analysis

Normality was tested with a Shapiro-Wilk test ( $p>0.05$ ) to analyze quantification data. Bartlett's test was also used to verify the homoscedasticity of the values ( $p>0.05$ ). A parametric test (ANOVA) was performed to compare data from all conditions (statistical significance  $p<0.05$ ). All statistical analyses and plots were performed with the open-source software program RStudio (Version 1.1.383 – © 2009-2017 RStudio, Inc., available from <https://rstudio.com>) using packages “ade4”, “agricolae”, “car”, “carData”, “ggplot2”, “GraphR” and “RVAideMemoire” (downloaded in 2020 from <https://cran.r-project.org/>).

## 3. Results and discussion

### 3.1. Opine detection by UHPLC-ESI-MS-QTOF

#### 3.1.1. A wide range of opines detected by UHPLC-ESI-MS-QTOF

Solutions of commercial or purified opines belonging to five opine families were prepared in UHPLC-MS grade water, and their ability to ionize and be detected by UHPLC-ESI-MS-QTOF was tested. All eighteen molecules were detected (Table 1), and all but agrociniopine A were observed with a major ion corresponding to the protonated molecule  $[\text{M}+\text{H}]^+$ , with sometimes the presence of the adduct ions  $[\text{M}+\text{Na}]^+$ ,  $[\text{M}+\text{K}]^+$  and/or the cluster ion  $[2\text{M}+\text{Na}]^+$ . Agrociniopine A was observed only in the form of ionic species  $[\text{M}+\text{Na}]^+$  and  $[\text{M}+\text{K}]^+$ . All these polar compounds were eluted between 0.845 and 1.440 min within our 10-min chromatography gradient. The accurate ion mass obtained for each opine was consistent with the theoretical mass calculated from the molecular formula of the corresponding ionic species. All the compounds were also submitted to MS/MS analyses to determine their characteristic  $\text{MS}^2$  fragmentation data (Table 1, and Fig.S1 in ESM2). Glucopinic acid was directly fragmented in the ESI source with a daughter ion at  $m/z$  148.0627 observed together with the  $[\text{M}+\text{H}]^+$  ion at  $m/z$  312.1321 in the MS spectrum; it thus appeared as a possible unstable opine. This fragment ion was suspected to correspond to the glutamic acid moiety of glucopinic acid (theoretical mass of the  $[\text{M}+\text{H}]^+$  ion: 148.06043, calculated for  $\text{C}_5\text{H}_{10}\text{NO}_4$ ; mass error, 15.3 ppm). To our knowledge, few opine fragmentation data



are available in the literature for LC-MS/MS analysis, but such data are useful for the identification of these compounds. For example, comparisons with MS/MS data of synthesized standard compounds were used to identify butylated opines (alanopine, lysopine, strombine, tauropine) in untargeted analysis of derivatized abalone extracts [18]. With similar product ions, our results are in agreement with their data from octopine standard fragmentation [18]. The MS/MS fragmentation data presented in Table 1 will thus be helpful to identify opines in further targeted or untargeted metabolomic investigations of plant extracts or other organisms like marine mollusks (known to produce opines) [17,18,31].

Each opine was then independently spiked in blank tomato matrix to evaluate whether they could also be detected within a complex mix of phytochemicals (*i.e.*, a plant extract). All opines were detected with retention times, accurate masses and MS/MS fragmentations similar to those obtained in the standard solutions. Thus, all natural opines tested were detected and identified by UHPLC-ESI-MS/MS-QTOF analysis in pure solution and in a plant extract without requiring prior derivatization.

A few synthetic non-natural opine analogs were also included. All of them were detected by UHPLC-ESI-MS/MS-QTOF analysis, in pure solution and spiked in blank tomato matrix, but the isomers exhibited retention times, accurate masses and MS/MS fragmentations similar to their natural opine analogs. Hence, as previously shown [59], our UHPLC-ESI-MS-QTOF method could also be used to monitor the bacterial degradation of each analogous compound independently spiked in culture broth.

### 3.1.2 Selectivity (specificity) of opine detection

To detect putative interfering compounds co-eluting with each opine, blank plant matrices were analyzed. Standard compound solutions and spiked blank plant matrices were also analyzed as controls. Based on the retention time and accurate mass expected for each opine, no potential interfering substance was found in the solvent blank or in the three blank plant matrices (extracts of tomato, kalanchoe, bird's-foot trefoil). Examples of typical chromatograms obtained from these investigations are shown in Fig.1 and Fig.S2 (ESM2), in which the targeted opines were nopaline and mannopine, respectively. To verify the absence of putative interfering substances in tumors (which are more complex than non-infected stem), the accurate mass of a given opine was searched for in an extract from a tumor that did not biosynthesize this opine (*e.g.*, in Fig.1-g nopaline was sought for in the extract of a tumor induced by an octopine-type strain). For each opine, no significant interference was detected in these tumor matrices. Thus, the selectivity (specificity) of the method was validated for all opines.

## 3.2. Validation of the quantification method

The quantification method was validated on nopaline, octopine and mannopine, using standard compounds available in the laboratory. These three opines represent different types of opine families commonly described in crown gall [21].

### 3.2.1. Linearity (Calibration curve)

Calibration curves were constructed by plotting the peak areas as a function of standard compound concentrations (ng/mL) and using a linear regression model. The calibration curves, correlation coefficients ( $R^2$ ) and linear ranges of each standard opine are shown in Table 2. Whatever the matrix, the calculated  $R^2$  values were higher than 0.995, therefore linearity was considered acceptable. The linear range was opine-dependent, and slightly narrower for nopaline. The slope of these calibration curves revealed that relative "area/concentration" responses were opine-dependent too, highlighting different ionizations of the compounds. For example, at a similar concentration, the ionization intensity of octopine was higher than those of nopaline and mannopine. Thus, opine quantification based on only one selected standard should be avoided.

### 3.2.2. Sensitivity (LOD and LOQ)

The LOD and LOQ of our method were expressed as the lowest detectable or quantifiable concentrations, respectively. The results in water solution and in 100-fold diluted blank tomato matrix are presented in Table 2.

Previous works on opine quantification methods usually report detection limits based on analyses of standard solutions in solvent and express the results as the lowest quantity of opine detected in the test. To our knowledge, LOQs have not been reported. Using an injection volume of 10  $\mu$ L of the standard solution in water, we detected 0.7 to 0.8 ng of opines (2.4 to 2.8 pmol, depending on the opine) and quantified at least 2.3 to 2.7 ng (8.1 to 9.3 pmol). Thus, our method was sensitive enough to quantify these opines. Several HPLC methods have been described for opine detection, with various detectors associated or not to a derivatization step specific to some opine types [17,34,37–39]. For example, LODs of 0.02  $\mu$ g for agropine and mannopine were obtained by HPLC coupled to a refractometer and without derivatization [38]. Our method is more sensitive. Zhang *et al.* [34] reported an HPLC method with fluorescence detection and prior derivatization with detection limits between 0.1 to 5 pmol for opines, among which nopaline, octopine and mannopine. Even though the authors did not give the precise value for each individual opine, our method displayed similar sensitivity with a LOD of 2.4–2.8 pmol with standard solutions, but without requiring a time-consuming derivatization step.

Other methods have been applied to quantify certain opines in crown gall or transformed plant tissues. Mannopine has been quantified between 0.5 to 2.3  $\mu$ g/cm<sup>2</sup> of tobacco leaf [34], whereas the amounts of nopaline and octopine have been suggested to be around 10–20 and 70–74 mg/g dry weight of potato and tomato tumor tissues, respectively [28]. Johnson *et al.* [60] showed that the amounts of octopine in tobacco tumors induced by various *A. tumefaciens* strains ranged from 0.75 to 6.25 mg/g of dry tissue, whereas another work reported between 0.024 and 0.411 mg/g dry weight of crown gall tissue in various plant species for octopine, and between 5.5 and 19.9 mg/g for nopaline [40]. Given these reported amounts of opine in infected plant tissue, the sensitivity of our method for nopaline, octopine and mannopine is adequate for routine detection and quantification requirements.

### 3.2.3. Intra- and inter-assay precision and accuracy

Precision (%RSD) and accuracy (%bias) results are shown in Table 3. Intra-assay precision and accuracy were 0.5–1.3% and 0.01–8.0%, respectively, whereas inter-assay precision and accuracy were 1.3–11.9% and 0.5–10.7%, respectively. For each opine and concentration level, precision and accuracy were below 15%. Therefore, the method was considered robust and reproducible [54,56].

### 3.2.4. Extraction efficiency (recovery)

The efficiency of opine extraction was determined by comparing the analytical signal obtained for each opine in a blank tomato matrix spiked with known amounts of opines before and after extraction. The results ranged between 103 and 108% (Table S2, ESM1).

### 3.2.5. Carry-over

The EIC of the targeted opines obtained from the solvent blank, analyzed right after analyzing a sample with a high opine concentration, did not reveal any peak indicating the presence of trace residues of these opines. Consequently, the method was considered reliable for routine analysis.

### 3.2.6. Stability

Opine stability during storage at -20°C for 12 days with freeze-thaw cycles between each analysis was assayed at low, medium and high concentrations. The %RSD values of the analytical signals were <15%, and

considered acceptable (Table S3, ESM1). At a concentration close to the LOQ, octopine proved to be the least stable opine with a %RSD of 14.96%. Investigations beyond 12 days at -20°C were not led.

### 3.2.7. Matrix effect

The effect of the matrix on opine ionization was evaluated directly in the crude extract (blank tomato matrix at 10 mg/mL) by comparing the peak area of the targeted opine in the spiked blank tomato matrix with the one obtained by injecting the neat standard solution at the same opine concentration. All opines were detected, but with analytical recoveries ranging between 5 and 20% according to the opine (data not shown). This crude extract concentration revealed an influence of the complex composition of the matrix on opine ionization (lower signal intensity) and pointed out the need to dilute the crude extract to reach precise quantification.

Then, the matrix effect was assessed by performing quantification tests with four concentrations of opines spiked in diluted blank tomato matrix. The quantifications were calculated from either a solvent-based or a matrix-based calibration curve, and the recovery rates were expressed as percentages of the expected values. The results for the three opines in 100-fold diluted blank tomato matrix (0.1 mg/mL) are presented in Table 4. These results showed good recovery rates for nopaline with both calibration curves. As for octopine and mannopine, quantification was only right and accurate when using the matrix-based calibration curve. Therefore, lower ionization resulting from a matrix effect of the crude extract can be solved by appropriately diluting the sample before quantitative analysis. Furthermore, matrix-based calibration curves have to be used if these three opines (nopaline, octopine and mannopine) have to be quantified. However, if nopaline is the only opine targeted in a tomato sample, it could be quantified precisely using a simple solvent-based calibration curve.

These tests were also performed with blank kalanchoe matrix, and similar results were obtained. They also showed the need to use a 100-fold dilution of the crude extract before quantitative analysis and a matrix-based calibration curve to obtain accurate results. In this case, the use of an appropriate dilution made it possible to avoid artificial enhancement of the signal. Linearity and matrix effect results for the blank kalanchoe matrix are presented in Table S4 and S5 (ESM1).

Using an appropriate dilution of the plant extract and a matrix-based calibration curve, opines present in plant tissues can be quantified accurately by UHPLC-ESI-MS-QTOF analysis. This could be useful for researchers to quantify opines produced in gall tissues or translocated to other plant parts [61].

## 3.3. Applications

### 3.3.1. Applications of the detection method: towards a new diagnostic tool

#### 3.3.1.1 Search for opines in tumors induced by strains harboring different types of Ti/Ri plasmids

Strains harboring different types of Ti/Ri plasmids were inoculated in tomato plants. Tumors were extracted 21 days post inoculation and analyzed to assess whether our UHPLC-ESI-MS-QTOF method indeed detected the expected opines according to literature data on opine synthase genes or previous reported opines (Table 5). Extracted ion chromatograms were generated based on the accurate masses of the putative ionic species of the expected opines. For each analysis, a solvent blank, a blank tomato matrix and extracts from tumors not expected to produce the targeted opines were used as controls to make sure that the detected signals were not caused by interfering substances. Standard solutions of the expected opines were also analyzed when available to confirm identification with a better confidence level [62]. Whatever the inoculated strain, at least one of the expected opines (if not all) was detected in the tumor extracts (Table 5).

Most of the opines with available standard compounds were detected in the tumors when their presence was expected, except in three cases: cucumopine was not detected in extracts from tumors induced by strains CFBP 2407 and CFBP 2736, and agrociniopine A was not detected in the tumor caused by strain C58. As all opines were detected when they were spiked in a plant extract (see above 3.1.1), several hypotheses can be

proposed. For instance, they may have been preferentially consumed by the bacteria inside the tumor or they may not have been produced (or in too small quantities) under our experimental conditions (incubation time, plant species ...). However, cucumopine was detected in the K599-induced tumor, which confirms that cucumopine can indeed be detected in an induced plant tumor. The result expected for strain NIAES 1724 – which induces mikimopine biosynthesis in hairy roots – was also confirmed. As cucumopine and mikimopine are diastereoisomers [50], they were not distinguished by MS analyses and showed similar retention times in our separation conditions. The use of a chiral column or another specific stationary phase [63,64] might separate them on the chromatogram. In any case, they are not expected to be produced concomitantly because their biosynthesis is driven by different genes currently described as being harbored by different Ri plasmids [64].

Even when standard opines were not available, we detected several expected opines based on the consistency of accurate mass vs. theoretical mass and on the absence of similar signals detected in any of the control analyses. Chrysopine, deoxy-fructosyl-glutamine (santhopine), succinamopine, vitopine and rideopine were thus unequivocally detected in the tumor extracts (Table 5). Two kinds of succinamopines (D,L- and L,L-succinamopines) encoded by different synthases have been described in tumors [65–67]. As they have the same molecular formula, they are indistinguishable by MS, and the one we detected was annotated with the generic term succinamopine. However, it was most likely to be D,L-succinamopine because the opine synthase gene sequence of the Ti plasmid of strain CFBP4424 (*susD* gene) is identical to the *susD* sequence of the Ti plasmid of the reference strain EU6 [67]. *Allorhizobium vitis* S4 strain induces vitopine (=heliopine) and rideopine biosynthesis by transformed plant cells [33]. These two compounds have the same molecular weight (218 Da), but owing to their different molecular formulae we differentiated them by UHPLC-ESI-MS-QTOF based on the accurate masses of their adduct ions  $[M+H]^+$ . Furthermore, vitopine MS/MS data confirmed this identification with a higher confidence level, consistent with its structure (Fig.S3, ESM2).

All these results demonstrate that our analytical method detected opines of different structural types in real tumors or hairy roots induced by pathogenic strains.

### 3.3.1.2 Diagnostic tool of tumors: the case of *Rosa* sp. plants suspected to have crown-gall disease

Two rose plants from a horticultural nursery with abnormal stem tissue outgrowths similar to crown-gall disease symptoms were subjected to diagnostic analyses to determine if these outgrowths were caused by pathogenic agrobacteria. For each rose plant, gall tissues were harvested, extracted and analyzed (at 10 mg/mL) by UHPLC-ESI-MS-QTOF, and the accurate masses of the putative ionic species of known opines were searched for in the MS data from each tumor extract.

Nopaline was detected in one of the two tumors (Fig. 2), and its identification was confirmed by standard compound analysis. Therefore, only the tumor containing nopaline was caused by pathogenic agrobacteria. The results of the conventional diagnostic process (with strain isolation on agrobacterium-selective medium followed by pathogenicity tests on model plants [69]) were consistent with the opine detection results: a pathogenic *Agrobacterium* was only isolated in the nopaline-positive tumor (data not shown). These results demonstrate that (i) opines can also be detected in rose tissue (another plant matrix), and (ii) our UHPLC-ESI-MS-QTOF method is an efficient diagnostic tool to identify abnormal tissue outgrowths caused by crown-gall disease.

## 3.3.2. Application of the quantification method

### 3.3.2.1 Influence of the At plasmid on the opine content of tumors induced by isogenic strains

The *A. fabrum* C58 strain contains a circular chromosome, a linear chromosome, and two large plasmids – the Ti and At plasmids [70,71]. The ecological role of the At plasmid (pAt) is not defined as precisely as that of the Ti plasmid (pTi) [72]. However, it may have a positive effect on virulence gene induction, and pAt-free strains have been reported to be less virulent (to induce the formation of smaller tumors) [46]. These observations led us to investigate if the presence of pAt had an impact on the opine content of tumors from

infected plants. Opines were quantified in non-inoculated plants and plants inoculated with the *A. fabrum* wild-type strain (C58), as well as with isogenic strains harboring or not pTiC58 and/or pAtC58 (strains UIA5, AB150, AB152 and AB153 described in ESM1 Table S1) [46]. Tumors or stem tissues were extracted 21 days post inoculation, and the crude extracts were diluted 100-fold and analyzed by UHPLC-ESI-MS-QTOF to detect and quantify the expected nopaline as described above. First, as expected, nopaline was not detected in the control samples (non-inoculated plants, and plants inoculated with the plasmid-less UIA5 strain and the AB150 strain harboring only pAtC58), confirming the absence of an interfering substance. Secondly, nopaline quantification in the tumors induced by the strains harboring pTiC58 and pAtC58 (strains C58 and AB153) or only pTiC58 (strain AB152) showed nopaline amounts ranging from 1.64 to 2.30  $\mu\text{g}/\text{mg}$  of crown gall tissue dry weight (Fig.3). These results are consistent with literature data. For instance, nopaline amounts of 10-20  $\mu\text{g}/\text{mg}$  of tumor dry weight were detected in tomato tumors 5-6 weeks post inoculation [28]. Finally, our results revealed significantly higher nopaline contents in the tumors of plants inoculated with the strains harboring both plasmids (AB153, C58) than in the tumors induced by strain AB152 that only harbored pTiC58 (Fig.3). In addition, the quantities of bacteria still persisting in the three types of tumors were similar (data not shown), indicating that variation of the opine content did not result from a difference in *A. fabrum* concentration in the tumor tissue. The lower nopaline abundance in the tumors induced by At plasmid-free agrobacteria is consistent with previous works suggesting that the At plasmid increases *A. fabrum* C58 virulence [46].

#### 4. Conclusion

In the present work, a general, rapid, specific and sensitive method was developed and validated for opine analysis. Using a simple process of sample preparation (solid-liquid tumor extraction, without time-consuming purification and/or derivatization steps) followed by UHPLC-ESI-MS-QTOF analysis, opines of different structural types were detected unequivocally in pure or mixed solutions, and in extracts of tumors/hairy roots induced by strains harboring various Ti/Ri plasmids. Detection and identification were based on the accurate mass of the precursor ion, unequivocal monoisotopic mass, accurate mass of MS/MS fragment ions, and the matching of MS spectra and retention times with data obtained from standard compounds when available. Moreover, our method was successfully used with several plant species (tomato, rose, kalanchoe, bird's-foot trefoil). Taking all these results into account, at least one molecule of each opine family commonly described in infected plants [15] was detectable. The nature of the opines produced by the abnormal tissue outgrowths depends on the plasmid harbored by the pathogenic strain. These opines constitute persistent biomarkers of plant infection by pathogenic agrobacteria. The UHPLC-ESI-MS-QTOF method presented in this study represents a powerful tool to perform diagnostic analyses of plant tumor tissue because it can detect a wide range of opines in one run. It could be easily implemented in an analytical chemistry platform. It will be useful for researchers and plant growers to determine if a gall observed on a plant is due to crown gall disease or not. As no curative methods are available for this plant disease, early diagnosis is essential for taking appropriate sanitation precautions to avoid dissemination and long-lasting contamination, and thus prevent pathogenic agrobacteria establishing in crops.

Based on this UHPLC-ESI-MS-QTOF method, a quantitative assay was also developed and validated for three selected opines representing different types of opine families commonly described in crown gall (nopaline, octopine and mannopine). With an appropriate dilution of the plant extract during sample preparation and the use of a matrix-based calibration curve, this method proved to be sensitive enough to allow for accurate and reproducible quantification of these opines in plant extracts (tomato and kalanchoe). The method was successfully applied to study the nopaline content of tumors induced by isogenic strains harboring the At plasmid or not, confirming the positive effect of this plasmid on *A. fabrum* C58 virulence highlighted in previous works. Quantitative monitoring of opines in plant samples offers the opportunity to accurately quantify opine

production in crown-gall tissues or hairy roots, or to evidence the spatiotemporal distribution of any given opine in an infected plant. Thus, from a more academic perspective, this method can also be a useful tool for researchers, for them to better understand these plant-pathogenic bacterium interactions.

### **Conflict of interest**

The authors declare that they have no conflict of interest.

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**Fig.1. Typical chromatograms of the search for nopaline in different types of samples**

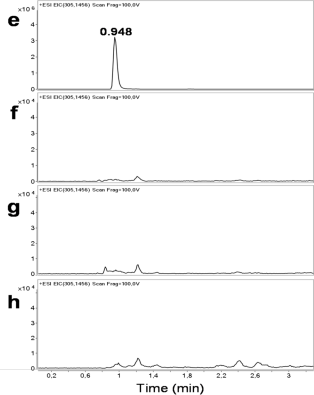
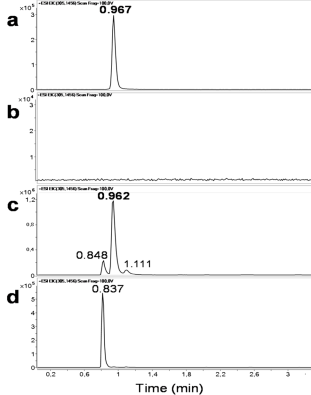
Each chromatogram is an extracted ion chromatogram (EIC) at  $m/z$  305.1456 (expected value for the ionic species  $[M+H]^+$  for nopaline). **a**- injection of a standard solution of nopaline at 500 ng/mL in water; **b**- injection of water used for preparing the samples and standard solutions (blank solvent injection); **c** and **d**- injection of crude extracts (10 mg/mL) of *K. daigremontiana* plant tissues: tumor induced by the C58 strain harboring a nopaline-type Ti plasmid (**c**), and non-inoculated plant stem tissue (blank kalanchoe matrix) (**d**); **e** to **h**- injection of crude extracts (10 mg/mL) of *Solanum lycopersicum* plant tissues: tumor induced by the C58 strain harboring a nopaline-type Ti plasmid (**e**), non-inoculated plant stem tissue (blank tomato matrix) (**f**), tumor induced by the C58 derivative strain (CFBP 1898) harboring an octopine-type Ti plasmid (**g**), and stem sample above the latter tumor (**h**). In **c** and **d**, the chromatographic peak with a retention time around 0.84 min corresponds to an ion at  $m/z$  305.1375 which is not nopaline, whereas in **a** and **e** the chromatographic peak around 0.96 min is an ion at  $m/z$  305.1490 consistent with nopaline. At the retention time of nopaline (observed in the standard solution analyses in the same sequence), no potential interfering substance was detected in the analyses of blank solvent injection (**b**) or of blank plant matrix for kalanchoe (**d**) and tomato (**f** and **h**) plants, or in extract of tumors induced by a strain harboring an octopine-type Ti plasmid not expected to induce nopaline biosynthesis in the tumor (**g**).

**Fig.2. Diagnostic analysis of tumors harvested on *Rosa* sp. plants suspected to have crown-gall disease**

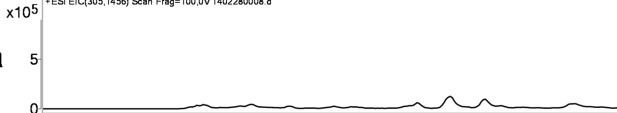
**a**, **b** and **c**- Extracted ion chromatograms (EICs) at  $m/z$  305.1456 (expected value for the ionic species  $[M+H]^+$  of nopaline) of the tumor crude extracts (at 10 mg/mL) from *Rosa* sp. plants (**a** and **b**) and of a standard solution of nopaline at 1 µg/mL in water (**c**). The spectra show the accurate mass of the ions detected in **b** and **c**.

**Fig.3. Box-plots illustrating the nopaline contents of tumors induced by isogenic strains harboring pAt or not**

Strains C58 (wild-type) and AB153 (isogenic to C58) harbored the pTiC58 and the pAtC58, while strain AB152 (isogenic to C58 without the pAtC58) contained only the pTiC58. Nopaline quantification was expressed in µg/mg of tumor dry weight. Boxes cover 50% of the data. Central lines represent the medians and whiskers represent the minimum and maximum values among non-atypical data. The cross (+) denotes the mean value of the data (n=6). Different letters indicate significant differences between strains (ANOVA,  $p < 0.05$ ).



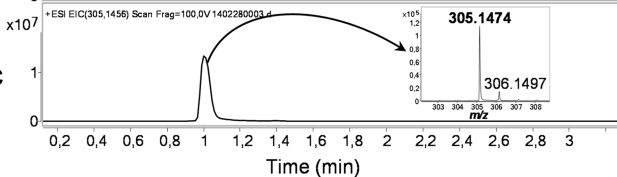
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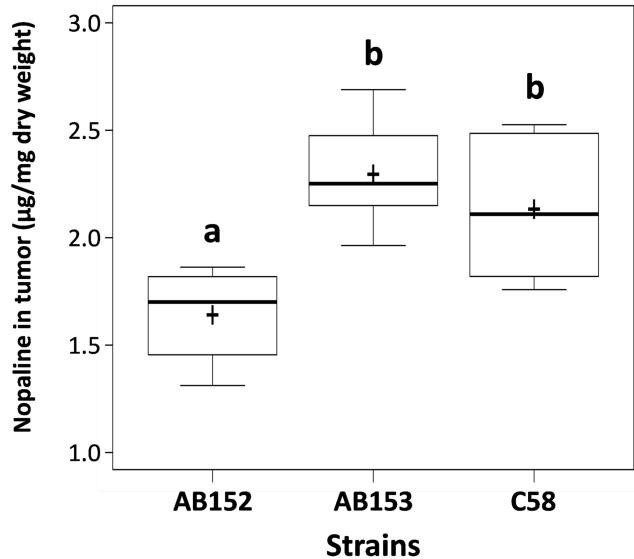


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+ESI EIC(305,1456) Scan Frag=100,0V 1402280003.d





**Table 1. Results of UHPLC-ESI-MS/MS-QTOF analysis of standard compounds belonging to different opine families.**

Opines		UHPLC-ESI-MS-QTOF analysis					UHPLC-ESI-MS/MS-QTOF analysis																			
Opine family	Compound	RT (min)	[M+H] <sup>+</sup> or [M+Na] <sup>+</sup>				Main product ions observed <i>m/z</i> (relative abundance)																			
			Measured <i>m/z</i>	Calculated <i>m/z</i>	Δppm	Molecular formula																				
Nopaline	Nopaline	0.970	305.1481	305.1456	8.3	C <sub>11</sub> H <sub>21</sub> N <sub>4</sub> O <sub>6</sub>	305(32)	287(28)	228(20)	200(100)	175(41)	158(24)	130(86)	70(66)	60(52)											
	Pyronopaline	1.440	287.1371	287.1350	8.4	C <sub>11</sub> H <sub>19</sub> N <sub>4</sub> O <sub>5</sub>	287(49)	270(6)	228(90)	227(27)	200(9)	184(10)	182(47)	181(41)	156(9)	142(7)	130(100)	114(9)	97(8)	95(8)	84(17)	71(9)	70(17)			
Cucumopine	Cucumopine	0.860	284.0905	284.0877	9.8	C <sub>11</sub> H <sub>14</sub> N <sub>3</sub> O <sub>6</sub>	238(8)	220(14)	205(100)	204(10)	194(16)	192(8)	177(57)	176(57)	163(7)	149(21)	148(39)	135(12)								
Octopine	Octopine	0.916	247.1419	247.1401	7.4	C <sub>9</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub>	230(3)	188(7)	175(12)	158(7)	142(100)	130(41)	114(13)	98(27)	70(39)	60(38)										
	Allo-octopine	0.917	247.1422	247.1401	8.6	C <sub>9</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub>	247(5)	188(7)	175(9)	158(5)	142(100)	130(36)	116(5)	114(11)	98(28)	87(10)	72(9)	70(38)	60(38)							
	Octopinic acid	0.845	205.1203	205.1183	9.8	C <sub>8</sub> H <sub>17</sub> N <sub>2</sub> O <sub>4</sub>	205(6)	188(30)	187(8)	142(100)	141(40)	116(11)	70(23)													
	Histopine	0.869	228.0999	228.0979	8.8	C <sub>9</sub> H <sub>14</sub> N <sub>3</sub> O <sub>4</sub>	228(44)	182(85)	138(100)	111(1)	95(33)	83(3)	68(1)													
	Alanopine	0.984	162.0775	162.0761	8.7	C <sub>6</sub> H <sub>12</sub> NO <sub>4</sub>	162(4)	116(100)	88(10)	84(5)	70(87)															
Mannityl-opine	Nor-mannopine	0.852	297.1319	297.1292	8.9	C <sub>10</sub> H <sub>21</sub> N <sub>2</sub> O <sub>8</sub>	297(23)	280(2)	238(27)	234(12)	220(12)	216(12)	192(11)	174(8)	164(25)	133(6)	111(6)	88(100)	69(17)	60(12)						
	Mannopine	0.872	311.1479	311.1449	9.4	C <sub>11</sub> H <sub>23</sub> N <sub>2</sub> O <sub>8</sub>	311(17)	294(5)	276(12)	222(13)	194(11)	182(41)	142(27)	130(100)	129(16)	114(17)	111(47)	98(26)	84(31)	81(10)	69(7)					
	Mannopinic acid	0.898	312.1325	312.1289	11.5	C <sub>11</sub> H <sub>22</sub> NO <sub>9</sub>	312(65)	294(9)	276(9)	266(100)	248(40)	230(16)	194(15)	164(16)	160(19)	156(12)	148(12)	130(48)	129(13)	128(14)	111(22)	102(20)	98(29)	84(74)	69(37)	61(13)
	Glucopine	0.873	311.1480	311.1449	10.0	C <sub>11</sub> H <sub>23</sub> N <sub>2</sub> O <sub>8</sub>	311(18)	294(4)	276(25)	258(13)	230(18)	222(19)	194(12)	182(48)	176(16)	164(8)	142(46)	130(100)	129(20)	114(27)	111(58)	98(20)	84(36)	81(15)	69(9)	
	Glucopinic acid	0.886	312.1321	312.1289	10.2	C <sub>11</sub> H <sub>22</sub> NO <sub>9</sub>	nd																			
	Galactopine	0.872	311.1480	311.1449	10.0	C <sub>11</sub> H <sub>23</sub> N <sub>2</sub> O <sub>8</sub>	311(17)	294(4)	276(11)	258(7)	230(15)	222(22)	194(14)	182(44)	176(15)	142(37)	130(100)	129(19)	114(27)	111(52)	98(26)	84(33)				
	Galactopinic acid	0.883	312.1319	312.1289	9.6	C <sub>11</sub> H <sub>22</sub> NO <sub>9</sub>	312(62)	294(12)	276(14)	266(100)	248(44)	230(17)	160(19)	148(18)	142(34)	130(36)	116(23)	114(61)	111(27)	98(36)	85(21)	84(63)	81(16)	69(26)	68(18)	
	Agropine	0.907	293.1370	293.1343	9.1	C <sub>11</sub> H <sub>21</sub> N <sub>2</sub> O <sub>7</sub>	293(38)	275(12)	257(6)	171(16)	153(20)	141(7)	129(39)	109(14)	95(37)	85(8)	83(100)	69(10)	61(7)							
	Agropinic acid	1.283	294.1208	294.1183	8.4	C <sub>11</sub> H <sub>20</sub> NO <sub>8</sub>	276(7)	258(9)	230(21)	222(30)	194(13)	184(13)	176(38)	172(19)	148(15)	142(61)	130(68)	129(32)	126(23)	114(75)	111(100)	98(6)	84(59)	83(36)	81(17)	69(20)
Agrocinopine	Agrocinopine A	0.951	577.1194	577.1140	9.3	C <sub>17</sub> H <sub>31</sub> O <sub>18</sub> PNa	577(100)	559(58)	541(16)	492(6)	445(16)	397(14)	379(33)	320(6)	253(73)	235(22)	216(6)									

**Table 2. Calibration curves, correlation coefficients, LOD, LOQ, and linear ranges of nopaline, octopine and mannopine in two matrices.** For each calibration curve, each point corresponds to the mean  $\pm$  SD (n=4 for the blank solvent; n=3 for blank tomato matrix). LOD, LOQ and linear range data are expressed in ng/mL and in nmol/mL so as to be easily compared with literature data.

Matrix	Opine	Calibration	R <sup>2</sup>	LOD		LOQ		Linear range	
				ng/mL	nmol/mL	ng/mL	nmol/mL	ng/mL	nmol/mL
Blank solvent (water)	Nopaline	$y = 1883x - 50975$	0.9984	74.3	0.24	247.8	0.81	248 - 2500	0.81 - 8.22
	Octopine	$y = 5541x - 225067$	0.9994	68.7	0.28	229.1	0.93	229 - 5000	0.93 - 20.30
	Mannopine	$y = 2514x - 52160$	0.9997	81.9	0.26	273.1	0.88	273 - 5000	0.88 - 16.11
Blank tomato matrix 100-fold diluted	Nopaline	$y = 1775x + 80364$	0.9982	134.7	0.44	449.0	1.48	449 - 2500	1.48 - 8.22
	Octopine	$y = 2418x + 212965$	0.9956	119.7	0.49	399.0	1.62	399 - 5000	1.62 - 20.30
	Mannopine	$y = 692x + 87112$	0.9966	78.5	0.25	261.7	0.84	262 - 5000	0.84 - 16.11

**Table 3. Results of intra- and inter-assay precision and accuracy of the UHPLC-ESI-MS-QTOF method developed to quantify opines.**

Compound	Expected concentration (ng/mL)	Intra-assay (n = 4)				Inter-assay (n = 3)			
		Concentration (ng/mL)			Precision	Accuracy	Concentration (ng/mL)		
		Mean	±	SD	%RSD	%bias	Mean	±	SD
Nopaline	1000	939.9	±	4.8	0.5	6.1	966.7	±	91.3
	750	727.6	±	8.6	1.2	3.0	728.6	±	36.8
	500	473.2	±	5.9	1.3	5.4	510.2	±	19.5
	250	239.4	±	1.2	0.5	4.2	261.0	±	26.7
Octopine	1000	948.0	±	5.4	0.6	5.2	936.2	±	74.7
	750	772.4	±	9.8	1.3	3.0	746.1	±	38.7
	500	487.4	±	3.1	0.6	2.5	486.9	±	58.1
	250	250.0	±	2.4	1.0	0.01	225.2	±	6.7
Mannopine	1000	1001.1	±	7.5	0.8	0.1	941.9	±	13.6
	750	809.9	±	5.1	0.6	8.0	712.9	±	9.0
	500	518.1	±	5.1	1.0	3.6	446.3	±	15.4
	250	244.7	±	1.0	0.4	2.1	274.0	±	6.7



**Table 4. Matrix effect assessment: recovery (%) of opine standards spiked into a diluted blank tomato matrix, calculated from solvent-based or matrix-based standard curves.** Mean recovery rates were expressed as percentages of the expected values, and relative standard deviations (% RSD) were calculated (n=3).

Opine	Blank plant matrix	Opine concentration (ng/mL)	Quantification			
			Solvent-based standard curve		Tomato matrix-based standard curve	
			(water)		(100-fold diluted)	
			Recovery (%)	%RSD	Recovery (%)	%RSD
Nopaline	Blank tomato matrix, 100-fold diluted	2500	99.0	6.2	96.2	6.6
		1000	109.3	7.3	105.4	7.4
		500	108.8	4.3	103.6	5.1
		250	105.0	2.8	97.1	0.8
Octopine	Blank tomato matrix, 100-fold diluted	2500	47.4	6.2	95.7	6.4
		1000	56.1	4.9	107.0	5.5
		500	62.9	2.1	109.7	2.7
		250	66.2	2.2	93.2	3.4
Mannopine	Blank tomato matrix, 100-fold diluted	2500	29.1	3.4	97.7	3.6
		1000	34.5	5.4	107.8	6.9
		500	39.0	2.7	108.3	4.3
		250	44.4	4.5	96.6	7.3

**Table 5. Detection of opines in extracts of tomato tumors induced by strains harboring diverse Ti/Ri plasmids.**

Bacterial species	Strain	Literature data		UHPLC-ESI-MS-QTOF analysis of tomato tumor extracts				
		Expected opines in the tumor	Reference	RT (min)	[M+H] <sup>+</sup>			Opine detected
					Measured <i>m/z</i>	Ion molecular formula	$\Delta$ ppm	
<i>Agrobacterium</i> sp.	CFBP 2407	Cucumopine, octopine	[43]	0.934	247.1422	C <sub>9</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub>	8.6	Octopine <sup>b</sup>
<i>Agrobacterium fabrum</i>	CFBP 2788	Chrysopine, nopaline, santhopine (DFG <sup>a</sup> ), DFOP <sup>a</sup>	[44]	1.178	291.1242	C <sub>11</sub> H <sub>19</sub> N <sub>2</sub> O <sub>7</sub>	18.9	Chrysopine
				1.020	305.1497	C <sub>11</sub> H <sub>21</sub> N <sub>4</sub> O <sub>6</sub>	13.6	Nopaline <sup>b</sup>
				0.940	309.1324	C <sub>11</sub> H <sub>21</sub> N <sub>2</sub> O <sub>8</sub>	10.2	Santhopine (DFG)
<i>Agrobacterium fabrum</i>	C58	Nopaline, agrocinopines A & B	[29,68]	0.939	305.1472	C <sub>11</sub> H <sub>21</sub> N <sub>4</sub> O <sub>6</sub>	5.4	Nopaline <sup>b</sup>
<i>Agrobacterium radiobacter</i>	CFBP 296	Nopaline	[47]	0.940	305.1518	C <sub>11</sub> H <sub>21</sub> N <sub>4</sub> O <sub>6</sub>	20.4	Nopaline <sup>b</sup>
<i>Agrobacterium</i> sp. G1	TT111	Octopine	[48]	0.872	247.1450	C <sub>9</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub>	19.9	Octopine <sup>b</sup>
<i>Agrobacterium</i> sp. G1	CFBP 2712	Agropine, mannopine	[43]	0.928	293.1400	C <sub>11</sub> H <sub>21</sub> N <sub>2</sub> O <sub>7</sub>	19.3	Agropine <sup>b</sup>
				0.894	311.1502	C <sub>11</sub> H <sub>23</sub> N <sub>2</sub> O <sub>8</sub>	17.1	Mannopine <sup>b</sup>
<i>Agrobacterium</i> sp. G3	CFBP 4424	Succinamopine	[47]	0.983	263.0900	C <sub>9</sub> H <sub>15</sub> N <sub>2</sub> O <sub>7</sub>	10.0	Succinamopine
<i>Allorhizobium vitis</i>	S4	Vitopine, rideopine	[33,49]	0.996	219.0987	C <sub>8</sub> H <sub>15</sub> N <sub>2</sub> O <sub>5</sub>	5.3	Vitopine
				0.837	219.1350	C <sub>9</sub> H <sub>19</sub> N <sub>2</sub> O <sub>4</sub>	4.9	Rideopine
<i>Allorhizobium vitis</i>	CFBP 2736	Cucumopine, octopine	[43]	0.962	247.1429	C <sub>9</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub>	11.4	Octopine <sup>b</sup>
<i>Rhizobium rhizogenes</i>	NIAES 1724	Mikimopine	[50]	0.935	284.0955	C <sub>11</sub> H <sub>14</sub> N <sub>3</sub> O <sub>6</sub>	27.4	Mikimopine / cucumopine <sup>b</sup>
<i>Rhizobium rhizogenes</i>	CFBP 2692	Agropine, mannopine	[43]	0.918	293.1419	C <sub>11</sub> H <sub>21</sub> N <sub>2</sub> O <sub>7</sub>	25.8	Agropine <sup>b</sup>
				0.895	311.1497	C <sub>11</sub> H <sub>23</sub> N <sub>2</sub> O <sub>8</sub>	15.5	Mannopine <sup>b</sup>
<i>Rhizobium rhizogenes</i>	K599	Cucumopine	[51]	0.935	284.0935	C <sub>11</sub> H <sub>14</sub> N <sub>3</sub> O <sub>6</sub>	20.4	Cucumopine / mikimopine <sup>b</sup>

<sup>a</sup> DFG = deoxy-fructosyl-glutamine = santhopine; DFOP = deoxy-fructosyl-oxo-proline

<sup>b</sup> These compounds were identified according to retention time and spectral data, and confirmed by comparison to the data of authentic standard obtained in the same sequence of analyses.