JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Physicochemical and Thermodynamic Characterization of the Encapsulation of Methyl Jasmonate by Natural and Modified Cyclodextrins Using Reversed-Phase High-Pressure Liquid Chromatography

José Manuel López-Nicolás,*[,†] Marta Escorial Camps,[†] Horacio Pérez-Sánchez,[‡,#] and Francisco García-Carmona[†]

[†]Department of Biochemistry and Molecular Biology A, Faculty of Biology, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain

[‡]Computer Science Department, Catholic University of Murcia (UCAM), 30107 Guadalupe, Spain

Supporting Information

ABSTRACT: Although the combinations of methyl jasmonate (MeJA) and cyclodextrins (CDs) have been used by different authors to stimulate the production of several metabolites, no study has been published about the possible formation of MeJA−CD complexes when these two molecules are added together to the reaction medium as elicitors. For this reason and because knowledge of the possible complexation process of MeJA with CD under different physicochemical conditions is essential if these two molecules are to be used in cell cultures, this paper looks at the complexation of MeJA with natural and modified CDs using a reversed-phase high-pressure liquid chromatography (RP-HPLC) system. The interaction of MeJA with β-CD was more efficient than with α- and γ-CDs. However, a modified CD, HP-β-CD, was the most effective of all of the CDs tested. Moreover, MeJA formed complexes with CD with a 1:1 stoichiometry, and the formation constants of these complexes were strongly dependent upon the temperature of the mobile phase used but not the pH. To obtain information about the mechanism of the affinity of MeJA for CD, the thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ were calculated. Finally, molecular modeling studies were carried out to propose which molecular interactions are established in the complexation process.

KEYWORDS: *Cyclodextrin, jasmonate, elicitor, encapsulation, cell culture, reversed-phase liquid chromatography*

1. INTRODUCTION

Because of the difficulty of synthesizing chemically a lot of molecules for commercial application, much effort is currently being directed at improving the production of bioactive secondary metabolites from natural plants for use in both the food and pharmaceutical industries.[1] Among the different strategies followed to reach this objective, the elicitation of plant cell cultures with molecules such as methyl jasmonate (MeJA) or cyclodextrins (CDs), used alone or combined, is one of the most reported.[2,3]

MeJA is a bioactive compound found in a great variety of higher plants with many important functions, such as a role in the defense mechanism against insect attack or in the control of the plant response to stimuli, such as mechanical stress.[4,5] Moreover, its known that the exogenous application of low MeJA concentrations can affect the biosynthesis of important plants and fruit components.[6−10]

CDs are cyclic oligosaccharides formed from six to eight D-glucopiranose residues bonded by α1→4 glucosidic bonds.[11] The molecule is torus-shaped, and because of the hydrophobic character of its internal cavity and hydrophilic exterior, CDs can trap apolar compounds of different nature, such as fatty acids, stilbenes, vitamins, etc., in the cavity to form highly water-soluble inclusion complexes.[12] For this reason and because CDs are able to increase both the solubility and bioavailability of different compounds with proven health properties, their use in the food [three types of natural CDs have generally recognized as safe (GRAS) status and have been approved recently for use as additives in the European Union (EU), and the corresponding E numbers assigned are E-457, E-459 and E-458, respectively], cosmetic, and pharmaceutical industries is increasing.[13] Moreover, in recent years, the use of CDs to enhance metabolite production in suspension cell cultures, where they act as elicitors, has also been reported.[3,14−16]

Although MeJA and CDs have been used to increase the yield of metabolites by their addition to cell cultures, the low yield obtained when those elicitors are added individually has led to different authors to include both MeJA and CDs together in the reaction medium. For example, Lijavetzky et al.[17] studied the synergistic effect of MeJA and CD on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures, finding that the combined treatment increased resveratrol production by 1 order of magnitude. These authors concluded that the effect of MeJA combined with a true and strong elicitor, such as CDs, on cell division could be responsible for the observed synergistic effect of both compounds on resveratrol production and the

Received: July 9, 2013
Revised: October 6, 2013
Accepted: October 12, 2013
Published: October 12, 2013

Table 1. Effect of Additives to the Mobile Phase on the Retention Time of MeJA[a]

	retention time (min)				
mobile phase (% H_2O)	no addition	0.5 mM β-CD	3.5 mM β-CD	3.5 mM D-glucose	24.5 mM D-glucose
60	40.6	39.7	33.5	40.6	40.6

[a]Flow rate, 1.50 ± 0.01 mL/min; temperature, 25.0 ± 0.1 °C.

expression of genes in the stilbene pathway. Furthermore, Durante et al.[18] observed that a combination of CDs and MeJA produced a 300-fold increase in artemisinin levels compared to untreated suspensions. Finally, Belchi-Navarro et al.[19] showed that methyl jasmonate increases silymarin production in *Silybum marianum* (L.) Gaernt cell cultures treated with β-CDs.

Unfortunately, concerning the action mechanism of these elicitors has led to difficulty in interpreting the results obtained in relation to the increase in the metabolite yield. Although several authors have attributed the increase in resveratrol production and that of other bioactive compounds to a synergistic effect between the two elicitors,[17] the possibility of that MeJA encapsulation by CDs when both molecules are present in the same reaction medium has not been taken into account.

If a CD−MeJA complex is formed, there is the possibility that three species could be involved in the metabolite stimulation process: free MeJA, free CD, and the MeJA−CD complex. Therefore, a synergistic effect of the two elicitors is not the only possibility to explain the increase in metabolite production in cell cultures observed when MeJA and CDs are added together. Indeed, any involvement on the part of a MeJA−CD complex could be due to the protection of MeJA within the CD internal cavity or the increase of solubility of MeJA in the presence of CDs, as demonstrated for other guest/host molecule interactions. Moreover, if encapsulation of MeJA by CD is true, knowledge of the encapsulation process will help understand the concentrations of both free and complexed MeJA present in the cell culture and how the production of metabolites could be improved. Finally, the solubility of MeJA in aqueous systems can be increased in the presence of CDs.

To date, no research has looked into the possibility of MeJA−CD complex formation. However, if MeJA and CDs are to be used in combination to stimulate the production of bioactive metabolites, the first step must be to investigate whether such complexes are formed, then to characterize the molecular nature of any inclusion process, to determine the stoichiometric coefficients and formation constants of the complexes (K_F) in different conditions, and finally to propose the molecular interactions established in the complexation process using molecular modeling.

2. MATERIALS AND METHODS

2.1. Chemicals. MeJA, α-CD, β-CD, γ-CD, 2-hydroxypropyl-β-CD (HP-β-CD), and 2- hydroxyethyl-β-CD (HE-β-CD) were purchased from Sigma (Madrid, Spain). Copper sulfate and anhydrous D-glucose were supplied by Prolabo (Fontenay-Sous-Bois, France). The methanol and water used in this study were of high-pressure liquid chromatography (HPLC) grade and were purchased from Scharlau Chemie S.A. (Barcelona, Spain) and J.T. Baker (Deventer, Netherlands), respectively. Binary mixtures of water/methanol, with methanol percentages of 20−50%, were used without further purification.

2.2. Equipment and Experimental Procedures. A total of 20 μL of MeJA (prepared at a concentration of 0.2% in methanol) was injected for HPLC analysis using a Merck-Hitachi L-6200 pump (Merck-Hitachi, Darmstadt, Germany) and a detector Shimadzu SPD-M6A ultraviolet (UV) diode array (Shimadzu, Kyoto, Japan). An avalaible LiChrospher RP18 reversed-phase column (Agilent, Waldbronn, Germany) (150 × 4 mm inner diameter, 5 μm particle size) was used.

For all experiments, the mobile phase flow rate was set and systematically controlled at 1.50 ± 0.01 mL/min and the UV detector was operated at 210 nm.

Mobile phases were prepared according to the following procedure. After the desired methanol/water mixture was obtained, an accurately weighed amount of CD was added to 250 mL of the same in a 500 mL volumetric flask. When total dissolution at an ambient temperature had been achieved, the remaining amount of solvent was added to reach a final mobile phase volume of 500 mL. Whenever the mobile-phase solution was changed, the column was first conditioned for at least 1 h with the new solution mixture at a flow rate of 1.0 mL/min.

The column void volume, t_0, was determined using reagent-grade copper sulfate solution (0.01 mg/mL) as described by Clarot et al.[20]

2.3. Temperature Studies. To study the effect of the temperature on the complexation process of MeJA by CDs, the temperatures ranging from 15 to 40 °C were selected. The thermodynamic relationship shown in eq 1 was used to determine the thermodynamic parameters, standard enthalpy and entropy of transfer of the MeJA, from the mobile phase to the CD

$$\ln K_F = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (1)$$

where K_F is the apparent formation constant of the inclusion complex, which is determined as discussed in section 3.5, T is the temperature in kelvin, R is the gas constant, and ΔH° and ΔS° are standard enthalpy and entropy changes of the complexes formed in the mobile phase. For a linear plot of ln K_F versus $1/T$, the slope and intercept are $-\Delta H^\circ/R$ and $\Delta S^\circ/R$, respectively. To determine the Gibbs free energy change for the interactions that take place during the inclusion process, the following equation was used:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

2.4. Molecular Modeling. The molecular structures used in this study were built manually using AutoDock tools[21] or derived from experimental data. The structure of α-CD was obtained experimentally by Ali et al.,[22] while the structures of γ- and β-CDs were extracted from the crystal structures of Protein Data Bank (PDB) codes 2ZYK and 3CGT. HE-β-CD and HP-β-CD models were built by adding hydroxylethyl and hydroxylpropyl groups to the β-CD model. Molecular docking calculations were carried out using default parameters in AutoDock Vina.[23] Hydroxylethyl and hydroxylpropyl groups of HE-β-CD and HP-β-CD were explicitly considered as flexible during docking simulations. Graphical representations of the docking results were prepared using PyMOL (Molecular Graphics System, version 1.3, Schrödinger, LLC).

3. RESULTS AND DISCUSSION

3.1. Selection of the Optimum Organic Modifier To Characterize MeJA−CDs Complexes in Reversed-Phase (RP)-HPLC Systems. Although works of several authors have reported that using CDs as additives in the mobile phases in RP-HPLC decreases the retention time of the guest molecule,[24] several factors, including the type of organic modifier, can influence retention behavior and modify the stability constants of the complexes formed. For this reason, the first step in our

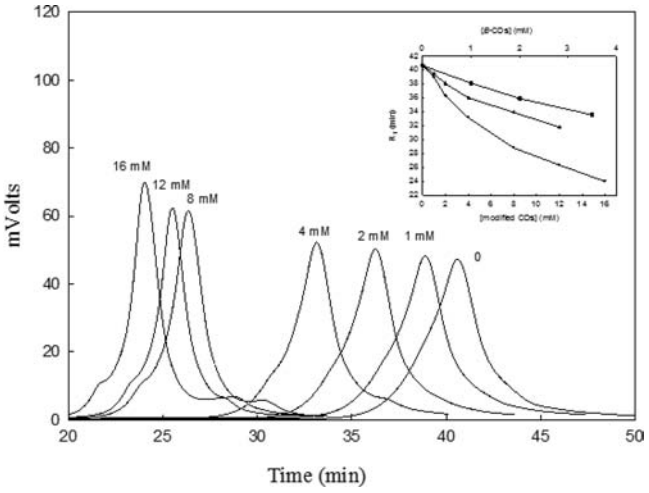

Figure 1. HPLC chromatograms at increasing HP-β-CD concentrations (0−16 mM) at 25 °C and with a methanol/water (40:60%) mobile phase (flow rate, 1.50 ± 0.01 mL/min). (Inset) Effect of increasing concentrations of CDs of β-CD (■), HP-β-CD (●), and HE-β-CD (▲) on the retention time of MeJA.

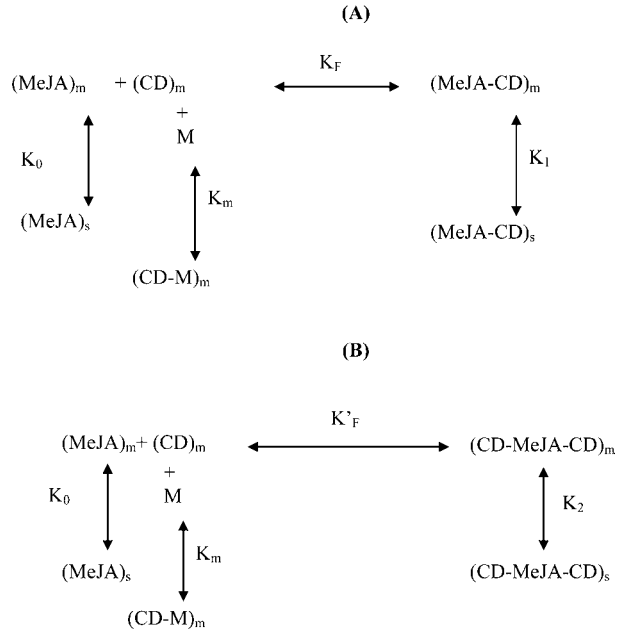

Figure 2. (A) Equilibria proposed for a 1:1 MeJA−CD inclusion complex. (B) Equilibria proposed for a 1:2 MeJA−CD inclusion complex. Abbreviations used: MeJA, methyl jasmonate; CD, cyclodextrin; MeJA−CD, methyl jasmonate−CD complex; CD−MeJA−CD, methyl jasmonate−$(CD)_2$ complex; m, mobile phase; s, stationary phase; M, organic modifier; K_m, affinity constant of the modifier for the CD cavity; K_F, formation constant for the MeJA−CD complex in a 1:1 model; K'_F, formation constant for the MeJA−CD complex in a 1:2 model; K_0, equilibrium constant of MeJA between mobile and stationary phases; K_1, equilibrium constant of the MeJA−CD complex between mobile and stationary phases; K_2, equilibrium constant of the MeJA−$(CD)_2$ complex between mobile and stationary phases; and CD−M, CD−organic modifier interaction.

investigation was to select the most suitable composition of the mobile phase for the analysis.

Although the formation of CD inclusion complexes in the liquid phase is more easy in an aqueous solution, an aqueous−organic solvent was used as a mobile phase in the present system because very long retention times were required for the analysis when water alone was used as the mobile phase.

To select the most appropriate organic solvent for encapsulating MeJA, two important parameters, the affinity of the organic modifier for the CD cavity and the solubility of CDs in the organic solvent, were studied because both strongly influence the chromatographic (retention value or the resolution of the sample) and encapsulating (binding constant of inclusion complexes of the solute) data.

Several types of organic solvent, such as ethanol, acetonitrile, or methanol, have been used with RP-HPLC to characterize the complexation of a guest molecule in CDs.[25] However, in this work, methanol was introduced in the corresponding mobile phases for two main reasons: (i) the solubility of CD in methanol is generally greater than in acetonitrile and tetrahydrofuran (THF), which permits the concentration of the β-CD in the mobile phase to be increased, thus improving characterization of the MeJA−β-CD complexes, and (ii) the association of methanol with β-CD (represented by the low value of K_m, the constant that describes the affinity of the organic modifier for the CD cavity) is very weak.[26] Indeed, the K_m value described for the interaction between methanol and β-CD (K_m = 0.32 M^{-1}) or α-CD (K_m = 0.93 M^{-1}) makes it a

Table 2. Apparent K_F Values and Correlation Coefficients Arising from Eqs 3 and 4 for Different MeJA−CD Complexes at 25 °C in a Methanol/Water (40:60%) Medium and Docking Scores Obtained from Simulations

		correlation coefficient		
complex	K_F (M^{-1})	1:1 using eq 3	1:2 using eq 4	docking score
MeJA−α-CD	30.4 ± 1.5	0.99	0.91	−5.9
MeJA−β-CD	60.9 ± 2.3	0.99	0.86	−6.8
MeJA−γ-CD	9.6 ± 0.08	0.99	0.92	−4.5
MeJA−HP-β-CD	43.1 ± 1.6	0.99	0.89	−6.4
MeJA−HE-β-CD	22.8 ± 1.1	0.99	0.89	−5.6

Table 3. Thermodynamic Parameters $\Delta H°$, $\Delta S°$, and $\Delta G°$ (at 25 °C) for the Association between MeJA and HP-β-CD for a Binary Mixture of Methanol/Water (40:60%, v/v)

complex	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J mol^{-1} K^{-1})	$\Delta G°$ (kJ/mol)
MeJA−HP-β-CD	−19.66	−33.72	−6.9

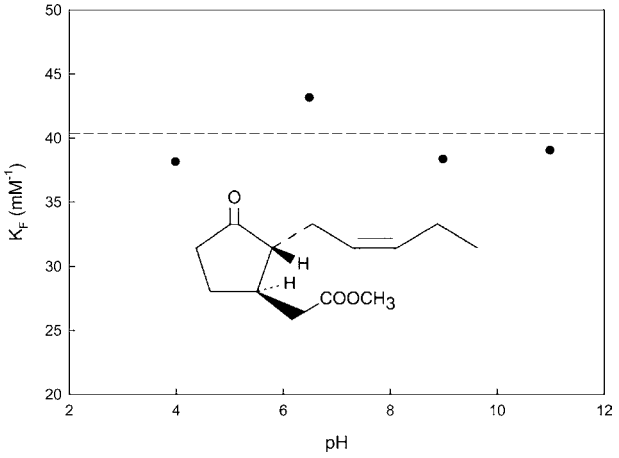

Figure 3. Apparent formation constant (K_F) of MeJA−CD complexes as a function of pH. (Inset) Structure of MeJA.

dx.doi.org/10.1021/jf402920p | J. Agric. Food Chem. 2013, 61, 11347−11354

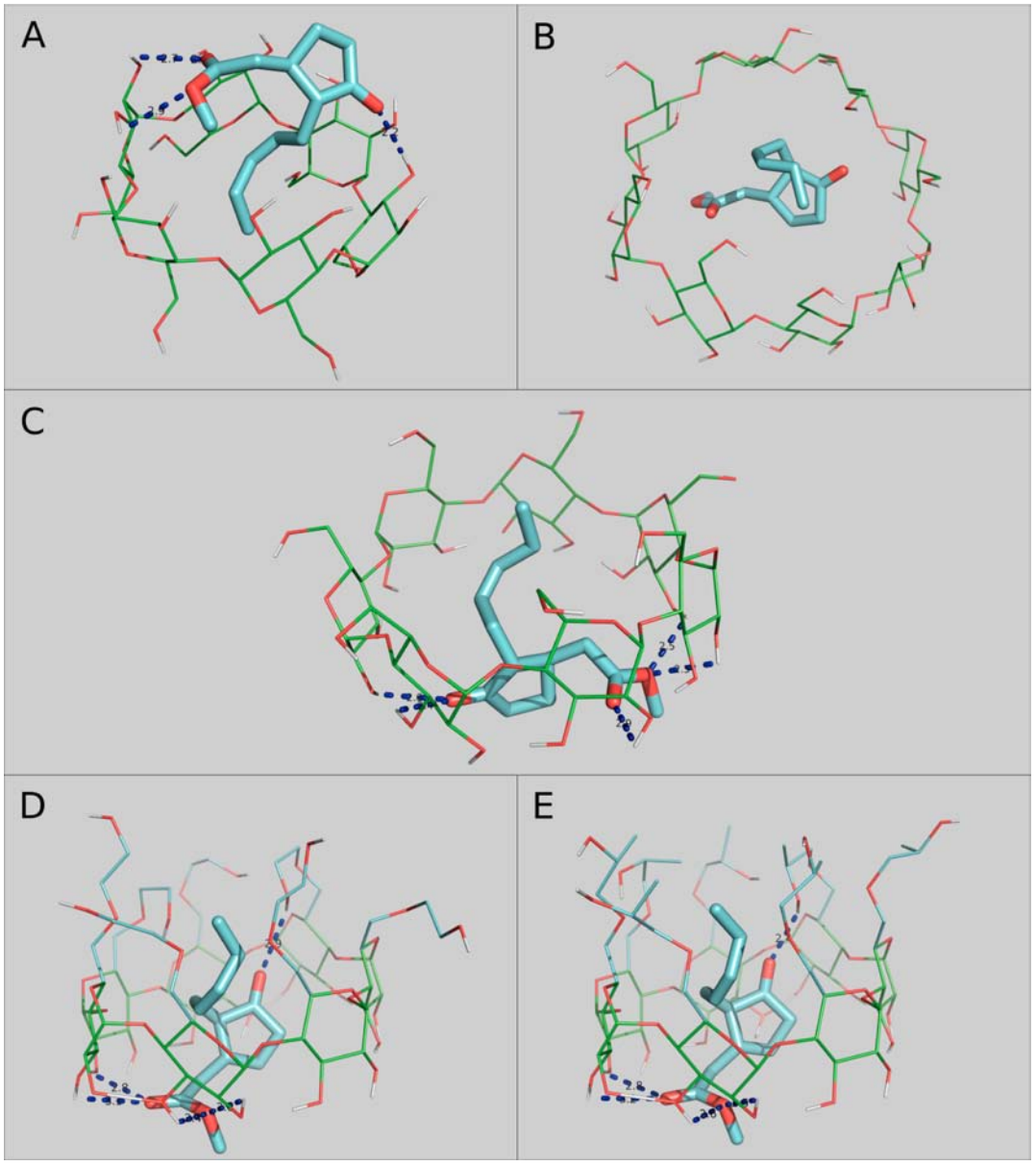

Figure 4. Docking results obtained in the complexation of MeJA with (A) α-CD, (B) γ-CD, (C) β-CD, (D) HE-β-CD, and (E) HP-β-CD. Hydrogen bonds are in dark blue. Carbon atoms of the flexible part of HE-β-CD and HP-β-CD are in light blue.

more favorable medium for MeJA−CD complexation than other alcohols, such as ethanol (K_m for β-CD = 0.93 M^{-1}, and K_m for α-CD = 5.62 M^{-1}) or 1-propanol (K_m for β-CD = 3.71 M^{-1}, and K_m for α-CD = 23.44 M^{-1}).

For these reasons, binary mixtures of methanol/water were used as the optimum composition of the mobile phase in RP-HPLC to study the complexation of MeJA by β-CD.

To calculate the optimum methanol concentration for studying the inclusion process, two factors were considered: the inclusion of methanol in the CD cavity and the analysis time. First, a substantial amount of methanol may interact with CD when a significant percentage of methanol is present in the mobile phase, leading to competition with MeJA (the association constant of methanol with β-CD is 0.32 M^{-1}).[26] Indeed, it has been demonstrated that methanol concentrations higher than 40% in binary methanol/water mixtures dramatically reduce the inclusion of guest molecules in CD. On the other hand, the concentration of the organic solvent in the mobile phase also influences the retention of the guest molecule. Indeed, in the presence of β-CD, MeJA retention increased with the increasing percentage of water. Because water concentrations higher than 60% led to very long retention times (longer than 75 min), with the associated experimental error, while water concentrations lower than 60% increased the competitive effect of methanol for the CD cavity, a 40:60% methanol/water mobile phase was selected.

3.2. Effect of the Addition of CDs to RP-HPLC System on the MeJA Retention. The next step in our investigation was to observe the effect of adding CDs to a binary mixture of methanol/water on MeJA retention. As shown in Figure S1 of the Supporting Information, when increasing concentrations of α-CD and HP-β-CD were added to the mobile phase, there was a significant reduction in the retention time (Rt) of MeJA, especially in the case of the modified CD.

However, although the data presented demonstrate that the addition of CDs to the mobile phase in RP-HPLC reduces the

retention of the guest molecule, a new experiment was necessary to confirm that this effect is due to a encapsulation phenomenon. To confirm that the potential effect of CDs on the *Rt* of the MeJA is due to the complexation ability and not the glucidic nature of CDs (glucose is a constituent of the CD molecule), we studied the effect of adding glucose and different CDs to the mobile phase on MeJA retention.

As observed in Table 1, two amounts of D-glucose (3.5 and 24.5 mM), corresponding to 0.5 and 3.5 mM of *β*-CD, respectively, in the number of glucose units, was added to a binary mixture of methanol/water (40:60%). The results show that the addition of 3.5 mM *β*-CD decreased the *Rt* of MeJA, whereas the presence of D-glucose did not alter the *Rt* values observed in the absence of any agent, even though the concentration of D-glucose was the same as that of *β*-CD with regard to the number of glucose units.

Two conclusions can be deduced from these data. First, the reduction in *Rt* values caused by the addition of CD to the mobile phase is due to the formation of an inclusion complex because no glucose/MeJA complexes were formed. Second, RP-HPLC appears to be a satisfactory method for observing and characterizing MeJA−CD inclusion complexes.

3.3. Effect of the Cyclodextrin Structure on the Complexation of MeJA. The next step was to study the interaction between MeJA and different types of natural and modified CDs with differing structures, sizes, and number of glucose units. First, three types of natural CD with GRAS status, all approved recently for use as additives by the EU (*α*-, *β*-, and *γ*-CDs), were used to this end. As shown in Figure S2 of the Supporting Information, increasing *α*-, *β*-, and *γ*-CD concentrations led to a reduction in the retention time of MeJA, with the lowest *Rt* being obtained with *β*-CD, followed by *α*- and *γ*-CDs.

These data mean that, at the molecular level, the inner diameter of the CD formed by seven units of glucose (*β*-CD, 6.0−6.4 Å) fitted MeJA better than an inner diameter of six units (*α*-CD, 4.7−5.2 Å) or eight units (*γ*-CD, 7.5−8.3 Å) of glucose. Because *β*-CD was the most effective CD for complexing MeJA, this natural CD was chosen to continue the investigation.

In recent years, there has been a sharp increase in the number of studies that use modified CDs for encapsulating different bioactive compounds. With regard to the use of CDs and MeJA to stimulate the production of different metabolites, such as resveratrol, silymarin, artemisinin, etc., hydroxylated and methylated CDs are among the modified CDs that have been used.

For this reason, we used HP-*β*-CD and HE-*β*-CD to characterize the encapsulation of MeJA by modified CDs. The chromatograms depicted in Figure 1 show that the addition of increasing concentrations (0−16 mM) of HP-*β*-CD reduced the *Rt* of MeJA from 40.6 to 24.0 min. This behavior is due to the formation of MeJA−HP-*β*-CD complexes that enhance MeJA solubility in the mobile phase and reduce its residency time in the column, leading to a significant decrease in the retention time of the guest molecule.

Moreover, in the inset of Figure 1, the *Rt* of MeJA in the presence of increasing concentrations of HP-*β*-CD and HE-*β*-CD is shown and compared to the data obtained with *β*-CD. Although this natural CD is able to encapsulate MeJA with a reasonable efficiency, its poor solubility in the mobile phase select was a problem.[13] For this reason, *β*-CD concentrations higher than 3.5 mM were not tested and this natural CD was discarded for the following steps of this investigation. Among the modified CDs tested, HP-*β*-CD reduced the *Rt* of MeJA with more efficacy than HE-*β*-CD. Moreover, the fact that HP-*β*-CD is highly soluble makes it the most appropriate CD for use in cell cultures.

3.4. Complexation Mechanism of MeJA by CD and Stoichiometry of the Encapsulation Process. In the previous sections, we have demonstrated that CDs are able to encapsulate MeJA. However, if MeJA−CD complexes are used in cell cultures as elicitors to stimulate the production of bioactive agents, it is absolutely necessary to quantify the interaction between these two compounds by determining the complexation constants (K_F). The determination of these constants will permit us to know the concentrations of both free and complexed MeJA present in the cell culture, and the production of metabolites will be improved.

We therefore determined the mechanism of complexation of MeJA by CD, the stoichiometry of the equilibria, and the apparent complexation constant between both guest and host molecules.

Modifications of the retention properties of molecules with different CD concentrations in the mobile phase were found to be related to the stoichiometry and stability of the inclusion complexes thus formed, as described by Gazpio et al.[24] Moreover, in other studies that have reported the competing equilibria in the column of a HPLC system upon introduction of the solute into a mobile-phase mixture consisting of CD and a primary organic modifier,[27,28] the presence of MeJA in the column in the presence of CD in the mobile phase was studied, obtaining the equilibria presented in panels A and B of Figure 2, for a 1:1 and 1:2 stoichiometry, respectively. As observed, when CD is added to the mobile phase, MeJA retention is governed by its partition between the mobile and stationary phases and the MeJA complexation with CD.

Assuming that the complex presents a 1:1 stoichiometry and that the interaction of the MeJA−CD complex with the stationary phase is negligible, we proposed to determine the K_F values for the MeJA−CD complexes as eq 3, which relates the capacity factor, *k*, and the CD mobile-phase concentration, [CD][25]

$$\frac{1}{k} = \frac{1}{k_0} + \frac{K_F}{k_0}[\mathrm{CD}] \tag{3}$$

where *k* is the capacity factor of the solute, k_0 is the solute capacity factor in the absence of CD, K_F is the apparent formation constant of the inclusion complex, and [CD] is the CD mobile-phase concentration.

Because MeJA may be complexed by two molecules of CD, we also studied the possible formation of a 1:2 MeJA−CD complex via a precursor 1:1 complex (Figure 2B). Equation 4 is an extension of eq 3 and includes a second-order term that accounts for the possibility of 1:2 MeJA−CD complex formation

$$\frac{1}{k} = \frac{1}{k_0}\frac{K'_F}{k_0}[\mathrm{CD}]^2 \tag{4}$$

where k_0 is the capacity factor of MeJA in the absence of CD modifier and K'_F is the apparent formation constant for the 1:1 and 1:2 MeJA−CD complexes.

To determine whether a molecule of MeJA is encapsulated by one or two molecules of CD, eq 3 was used to plot the reciprocal of *k* versus [CD]. A straight line would indicate the

formation of a 1:1 MeJA−CD complex. However, in the case of a 1:2 MeJA−CD complex formation, a plot of the reciprocal of k versus [CD] should give a parabolic curve that fits eq 4.

To determine the stoichiometry of MeJA−CD complexes, we selected the three natural CDs (α-, β-, and γ-CDs). As shown in Figure S3A of the Supporting Information, a plot of $1/k$ versus [CD] gave, in all three cases, a straight line with a linear correlation higher than 0.99, indicating that the presumed stoichiometry of the MeJA−CD complexes formed was 1:1. On the other hand, when $1/k$ was plotted against $[CD]^2$, a nonlinear relationship was obtained in the three cases (see Figure S3B of the Supporting Information), which indicates that the stoichiometry of the inclusion complex is not 2:1.

3.5. Determination of the Apparent Encapsulation Constant (K_F) Values of the MeJA−CD Complexes. The stability of the encapsulation process is given by the K_F values for CD−MeJA complexation. For this reason and because the K_F values between MeJA and CD reveal the amount of MeJA complexed in equilibrium with free MeJA, we calculated these constants in different conditions, such as different temperatures or pH values, that are known to influence the production metabolites in cell cultures and that may modify the inclusion process.

We have previously demonstrated that, in all of the conditions tested, MeJA forms complexes with CD of 1:1 stoichiometry. The equilibrium of 1:1 was therefore used to calculate the K_F values for MeJA complexes with all types of CD (natural and modified).

As shown in Table 2, for all of the CDs tested, a linear correlation of 0.99 was obtained, confirming the presumed 1:1 stoichiometry of the MeJA−CD complexes. However, when the 1:2 model was used, the linear correlation was very poor, so that the presence of complexes with a stoichiometry of 1:2 was discarded. Table 2 illustrated the differences between the K_F values obtained for all MeJA−CD complexes analyzed in this investigation. As seen, β-CD provided the highest K_F value. However, the used of the modified HP-β-CD is recommended because of its high solubility, as mentioned previously.

3.6. Effect of the Temperature on the Complexation of MeJA by HP-β-CD. Different studies have reported that the temperature is one the most important parameters that must be controlled in cell cultures because of its influence on the optimal production of metabolites. Moreover, several studies have demonstrated the dramatic changes that occur in the equilibrium between CD and different compounds when the temperature of the medium varies. However, the results obtained for the effect of the temperature on the K_F values are contradictory. While some authors found that an increase in the temperature of the system leads to an increase in the constant values, as is the case of the fatty acid−CD complexes,[12] others found that a decrease in the system temperature causes a dissociation of these complexes.[29]

For these reasons, we studied the effect of the temperature on the complexation of MeJA with HP-β-CD, determining the K_F values for temperatures of interaction between 15 and 40 °C. For all of the temperatures tested and for a binary mixture of methanol/water (40:60%) as the mobile phase, the stoichiometry of the MeJA−HP-β-CD complexes was 1:1, with the reciprocal of k for MeJA versus [HP-β-CD] showing a correlation coefficient higher than 0.99 (data not shown). From these data, different K_F values were obtained for different temperatures (see Figure S4 of the Supporting Information) and the degree of complexation of MeJA by HP-β-CD decreased as the temperature increased from 15 to 40 °C.

3.7. Thermodynamic Parameters for the MeJA−HP-β-CD Complexes. To study mechanistic aspects of the affinity of MeJA for HP-β-CD, the next step was to study the main thermodynamic parameters of the complexation process ($\Delta H°$, $\Delta S°$, and $\Delta G°$, at 25 ± 0.2 °C). For this, a van't Hoff plot (eq 1) was used to plot the ln K_F versus $1/T$. The data showed a linear representation, with a correlation coefficient higher than 0.99 (see the inset of Figure S4 of the Supporting Information).

From the results obtained (Table 3), three main conclusions can be drawn concerning the process of the complexation of MeJA by HP-β-CD: (i) Because of a decrease in the translational and rotational degrees of freedom of the complexed MeJA compared to the free MeJA, the process presents a negative value for entropy changes. (ii) The process is exothermic, as demonstrated by the negative values obtained for changes in enthalpy. This behavior is typical of hydrophobic interactions, van der Waals interactions, the displacement of water molecules from the cavity of HP-β-CD, and the formation of hydrogen bonds. (iii) The negative value obtained for the Gibbs free energy for the interactions that take place during the inclusion process at 25 ± 0.2 °C indicate that the process is spontaneous.

3.8. Effect of pH on the Apparent Formation Constants of the MeJA−HP-β-CD Complexes. One of the parameters that must be controlled in a cell culture is the pH of the reaction medium. For this reason and because of the reported effect of pH on the encapsulation process of some guest molecules, the last step of this work was to study the K_F values of the MeJA−HP-β-CD complexes at different pH values.

Although several papers have reported the strong role of pH in the complexation of some compounds, the basification of the reaction medium from pH 4.0 to 11.0 (Figure 3) did not lead to changes in the K_F values. This behavior may have been due to the absence of any functional group in the structure of MeJA that may be protonated or deprotonated by changes in the pH medium, as described for other molecules encapsulated by CDs, such as resveratrol, pinosylvin, pterostilbene, or oxyresveratrol.[30−33]

3.9. Molecular Modeling of the MeJA−CD Complexes. To understand how MeJA interacts with the different CD complexes, docking simulations were carried out. Table 2 shows that the docking scores were directly proportional to the Gibbs free energy of the complexation process and, thus, the K_F values. A good correlation between computed scores and experimental values can be observed. This might indicate that our modeling methodology captures the essentials of the host−guest energetic interactions. Additionally, the structural information about the different binding poses obtained by docking and shown in Figure 4 might explain experimental data obtained. With regard to the ring size of the different CDs, the weakest interaction corresponds to the complex with γ-CD, as depicted in Figure 4B. The main reason is the weak hydrophobic stabilization because of the poor host−guest fit. The α-CD is more stable (Figure 4A) because of the hydrogen bonds established with the primary hydroxy rim, but in general terms, the stability of the complexation increases with β-CD and its derivatives HE-β-CD and HP-β-CD (panels C−E of Figure 4) because the MeJA−CD fit is better. For CDs with seven sugar rings, the most stable situation corresponds to β-CD, followed by its derivatives HE-β-CD and HP-β-CD. In

these last cases, there might be less entropic stabilization than in β-CD because of the loss of degrees of freedom of the additional hydroxyethyl and hydroxypropyl groups. With regard to energy contributions, the reason for the greater stability of HP-β-CD with respect to HE-β-CD could be due to the additional hydrophobic stabilization of the extra methyl groups of HP-β-CD, which can point to the inner cavity.

In conclusion, although the combined use of MeJA and CD has increased in recent years in an attempt to stimulate the production of secondary metabolites, no research into the possible interaction of these two elicitors has been carried out. In this work, we demonstrate, for first time, that CDs are able to encapsulate MeJA, suggesting a new explanation for the increased yield of several metabolites when these elicitors are added together to cell cultures and not individually. The results obtained by adding CDs to mobile phases in a RP-HPLC system show that, although the stoichiometry of the complex is 1:1 for all of the conditions used, the K_F values for the MeJA−CD complexes are strongly dependent upon several factors, including the temperature or type of CD. A study of the main thermodynamic parameters of the complexation process shows that the encapsulation of MeJA by CDs is spontaneous and exothermic and presents a negative value for entropy changes. Finally, molecular docking calculations provide insights into how the different interactions at the molecular level (hydrophobic, hydrogen bonds, and van der Waals) influence the complexation constant.

■ ASSOCIATED CONTENT

Supporting Information

Effect of increasing concentrations of α-CD and HP-β-CD on the retention time of MeJA at 25 °C and with a methanol/water (40:60%) mobile phase (Figure S1), effect of increasing concentrations of natural CDs (α-, β-, and γ-CDs) on the retention time of MeJA at 25 °C and with a methanol/water (40:60%) mobile phase (Figure S2), reciprocal plots of MeJA complexed with α-, β-, and γ-CDs with a methanol/water (40:60%) mobile phase for determining the stoichiometry of MeJA−β-CD complexes (Figure S3), and apparent formation constant (K_F) of MeJA−HP-β-CD complexes as a function of the temperature (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Telephone: 34-868-8834777. Fax: 34-868-364147. E-mail: josemln@um.es.

#To whom all molecular modelling related correspondence should be addressed. Telephone: +34-968277982. E-mail: perez@ucam.edu.

Funding

This work was supported by AGL2011-25023 (MCI, FEDER, Spain) and "Programa de Ayudas a Grupos de Excelencia de Región de Murcia" of the Fundación Séneca (Plan Regional de Ciencia y Tecnología 2007/2010).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Lattanzio, V.; Kroon, P. A.; Linsalata, V.; Cardinali, A. Globe artichoke: A functional food and source of nutraceutical ingredients. *J. Funct. Foods* **2009**, *1*, 131−144.

(2) Repka, V.; Fischerová, I.; Šilhárová, K. Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell-suspension cultures. *Biol. Plant.* **2004**, *48*, 273−283.

(3) Morales, M.; Bru, R.; García-Carmona, F.; Barceló, A. R.; Pedreño, M. A. Effect of dimethyl-β-cyclodextrins on resveratrol metabolism in Gamay grapevine cell cultures before and after inoculation with shape *Xylophilus ampelinus*. *Plant Cell, Tissue Organ Cult.* **1998**, *53*, 179−187.

(4) Kessler, A.; Baldwin, I. T. Plant responses to insect herbivory: The emerging molecular analysis. *Annu. Rev. Plant Biol.* **2002**, *53*, 299−328.

(5) Weiler, E. W.; Albrecht, T.; Groth, B.; Xia, Z.-Q.; Luxem, M.; Liß, H.; Andert, L.; Spengler, P. Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of *Bryonia dioica*. *Phytochem.* **1993**, *32*, 591−600.

(6) Moreno, F.; de la, P.; Blanch, G. P.; Ruiz del Castillo, M. L. (+)-Methyl jasmonate-induced bioformation of myricetin, quercetin and kaempferol in red raspberries. *J. Agric. Food Chem.* **2010**, *58*, 11639−11644.

(7) Pérez, A. G.; Sanz, C.; Richardson, D. G.; Olías, J. M. Methyl jasmonate vapor promotes β-carotene synthesis and chlorophyll degradation in Golden Delicious apple peel. *J. Plant Growth Regul.* **1993**, *12*, 163−167.

(8) Wolucka, B. A.; Goossens, A.; Inzé, D. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* **2005**, *56*, 2527−2538.

(9) Tassoni, A.; Durante, L.; Ferri, M. Combined elicitation of methyl-jasmonate and red light on stilbene and anthocyanin biosynthesis. *J. Plant Physiol.* **2012**, *169*, 775−781.

(10) Lalel, H. J. D.; Singh, Z.; Tan, S. C. The role of methyl jasmonate in mango ripening and biosynthesis of aroma volatile compounds. *J. Hortic. Sci. Biotechnol.* **2003**, *78*, 470−484.

(11) Tegge, G.; Szejtli, J. Cyclodextrins and their inclusion complexes. *Starch/Staerke* **1982**, *34*, 395−395.

(12) López-Nicolás, J. M.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. Use of "soluble lipids" for biochemical processes: Linoleic acid−cyclodextrin inclusion complexes in aqueous solutions. *Biochem. J.* **1995**, *308*, 151−154.

(13) Del Valle, E. M. M. Cyclodextrins and their uses: A review. *Proc. Biochem.* **2004**, *39*, 1033−1046.

(14) Bru, R.; Sellés, S.; Casado-Vela, J.; Belchí-Navarro, S.; Pedreño, M. A. Modified cyclodextrins are chemically defined glucan inducers of defense responses in grapevine cell cultures. *J. Agric. Food Chem.* **2006**, *54*, 65−71.

(15) Zamboni, A.; Vrhovsek, U.; Kassemeyer, H.-H.; Mattivi, F.; Velasco, R. Elicitor-induced resveratrol production in cell cultures of different grape genotypes (*Vitis* spp.). *Vitis* **2006**, *45*, 63−68.

(16) Zamboni, A.; Gatto, P.; Cestaro, A.; Pilati, S.; Viola, R.; Mattivi, F.; Moser, C.; Velasco, R. Grapevine cell early activation of specific responses to DIMEB, a resveratrol elicitor. *BMC Genomics* **2009**, *10*, 363.

(17) Lijavetzky, D.; Almagro, L.; Belchi-Navarro, S.; Martínez-Zapater, J. M.; Bru, R.; Pedreño, M. A. Synergistic effect of methyljasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. *BMC Res. Notes* **2008**, *1*, 132.

(18) Durante, M.; Caretto, S.; Quarta, A.; De Paolis, A.; Nisi, R.; Mita, G. β-Cyclodextrins enhance artemisinin production in *Artemisia annua* suspension cell cultures. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1905−1913.

(19) Belchi-Navarro, S.; Pedreño, M. A.; Corchete, P. Methyl jasmonate increases silymarin production in *Silybum marianum* (L.) Gaernt cell cultures treated with β-cyclodextrins. *Biotechnol. Lett.* **2011**, *33*, 179−184.

(20) Clarot, I.; Clédat, D.; Battu, S.; Cardot, P. J. Chromatographic study of terpene derivatives on porous graphitic carbon stationary phase with β-cyclodextrin as mobile phase modifier. *J. Chromatogr., A* **2000**, *903*, 67−76.

(21) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30*, 2785−2791.
(22) Ali, S. M.; Khan, S.; Crowyn, G. Structure determination of fexofenadine−α-cyclodextrin complex by quantitative 2D ROESY analysis and molecular mechanics studies. *Magn. Reson. Chem.* **2012**, *50*, 299−304.
(23) Trott, O.; Olson, A. J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455−461.
(24) Gazpio, C.; Sánchez, M.; García-Zubiri, I. X.; Vélaz, I.; Martínez-Oharriz, C.; Martín, C.; Zornoza, A. HPLC and solubility study of the interaction between pindolol and cyclodextrins. *J. Pharm. Biomed. Anal.* **2005**, *37*, 487−492.
(25) López-Nicolás, J. M.; Núñez-Delicado, E.; Pérez-López, A. J.; Barrachina, A. C.; Cuadra-Crespo, P. Determination of stoichiometric coefficients and apparent formation constants for β-cyclodextrin complexes of *trans*-resveratrol using reversed-phase liquid chromatography. *J. Chromatogr., A* **2006**, *1135*, 158−165.
(26) Matsui, Y.; Mochida, K. Binding forces contributing to the association of cyclodextrin with alcohol in an aqueous solution. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 2808−2814.
(27) Debowski, J.; Jurczak, J.; Sybilska, D.; Zukowski, J. Separation of some aromatic amino acids by reversed-phase high-performance liquid chromatography using α- or β-cyclodextrin as mobile phase component. *J. Chromatogr., A* **1985**, *329*, 206−210.
(28) Muñoz de la Pena, A.; Ndou, T. T.; Anigbogu, V. C.; Warner, I. M. Solution studies of β-cyclodextrin−pyrene complexes under reversed-phase liquid chromatographic conditions: Effect of alcohols as mobile-phase comodifiers. *Anal. Chem.* **1991**, *63*, 1018−1023.
(29) Hinze, W. L. Applications of cyclodextrins in chromatographic separations and purification methods. *Sep. Purif. Rev.* **1981**, *10*, 159−237.
(30) López-Nicolás, J. M.; García-Carmona, F. Rapid, simple and sensitive determination of the apparent formation constants of *trans*-resveratrol complexes with natural cyclodextrins in aqueous medium using HPLC. *Food Chem.* **2008**, *109*, 868−875.
(31) López-Nicolás, J. M.; Rodríguez-Bonilla, P.; García-Carmona, F. Complexation of pinosylvin, an analogue of resveratrol with high antifungal and antimicrobial activity, by different types of cyclodextrins. *J. Agric. Food Chem.* **2009**, *57*, 10175−10180.
(32) López-Nicolás, J. M.; Rodríguez-Bonilla, P.; Méndez-Cazorla, L.; García-Carmona, F. Physicochemical study of the complexation of pterostilbene by natural and modified cyclodextrins. *J. Agric. Food Chem.* **2009**, *57*, 5294−5300.
(33) Rodríguez-Bonilla, P.; López-Nicolás, J. M.; García-Carmona, F. Use of reversed phase high pressure liquid cromatography for the physicochemical and thermodynamic characterization of oxyresveratrol/β-cyclodextrin complexes. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2010**, *878*, 1569−1575.