Development and validation of an HPLC method to quantify 2-Keto-3-deoxy-gluconate (KDG) a major metabolite in pectin and alginate degradation pathways

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Biomolecule platform

Abstract

A rapid and sensitive High Performance Liquid Chromatography (HPLC) method with UV and fluorescence detection is developed for routine analysis of 2-Keto-3-deoxy-gluconate (KDG), a catabolite product of pectin and alginate. These polysaccharides are both used for biofuel production and to generate high-value-added products. HPLC is performed, after derivatization of the carbonyl groups of the metabolite with *O*-phenylenediamine (OPD), using a linear gradient of trifluoroacetic acid and acetonitrile. Quantification is accomplished with an internal standard method. The gradient is optimized to distinguish KDG from its close structural analogues such as 5-keto-4-deoxyuronate (DKI) and 2,5-diketo-3-deoxygluconate (DKII). The proposed method is simple, highly sensitive and accurate for time course analysis of pectin or alginate degradation.

Keywords: KDG, HPLC, *O*-phenylenediamine, fluorescence, pectin or alginate degradation

Introduction

The major obstacle to use plant biomass for the production of fuels, chemicals, and bioproducts, is our current lack of knowledge of how to effectively deconstruct cell-wall polymers for their subsequent use as feedstocks. Pectin plays an important role in biomass recalcitrance [1]. Pectins are complex branched polysaccharides containing high amount of partly methyl-esterified galacturonic acid and low amount of rhamnose and carry arabinose and galactose as major neutral sugars. Due to their structural complexity, they are modifiable by many different enzymes, including polygalacturonases, pectate lyases, and esterases. Pectin-degrading enzymes and -modifying enzymes may be use in a wide variety of applications to modulate pectin properties or produce pectin derivatives for food industry [2], as well as for pharmaceutical industry. Pectin and pectin-derived oligosaccharides acts as anti-metastatic [3], cholesterol inhibiting [4], prebiotic and probiotic encapsulant [5]. In addition, pectin is being widely used as a component of bio-nano-packaging [6, 7].

In this context, numerous plant pathogenic microorganisms such as soft-rot bacteria (*Dickeya* species and *Pectobacterium*species) or necrotic fungi (*Aspergillus species, Botritys species*) are effective in pectin degradation and represent a large resource of pectinases that generated KDG metabolite [8-10]. In addition, novel metabolic pathways for hexuronate utilisation in various proteobacteria have been recently described and all converge to the KDG metabolite formation [11, 12].

Similarly, algae are a large group of marine vegetation that also immerge as feedstock for biofuel production [13, 14]. Alginate is a unique structural polysaccharide in algae, abundant in the cell wall ensuring resistance to mechanical stress. It is a linear copolymer of α-L-guluronate and its C5 epimer β-D-mannuronate [15]. Alginate degrading enzymes include different types of alginate lyases generating various alginate oligosaccharides which are further converted to KDG. Alginate lyases are present in a wide range of marine and terrestrial bacteria [16]. In addition to biofuel production, alginate derivatives can be used as therapeutic agents such as anticoagulants [17] and tumor inhibition [18]. Furthermore, alginate lyase also shows great potential application in treatment of cystic fibrosis by degrading the polysaccharide biofilm of bacterium [19]. KdgF was identifed as the missing link in the microbial metabolism of uronate sugars from pectin and alginate|20] (Figure 1)

Therefore, quantification of KDG is of wide interest to analyse the metabolic flux of hexuronate, pectin and alginate from numerous microorganisms.

In this paper, we took advantage of our expertise on the plant pathogenic bacteria *Dickeya dadantii* and the availability of both chemical and genetic resources in order to develop methods for monitoring and optimizing the breakdown of pectic polymers into oligomers or monomers of interest. This bacterium attacks a wide range of plant species, including many crops of economic importance. Soft rot, the visible symptom, is mainly due to the degradation of pectins by pectate lyase (Pel) activity generating unsaturated oligogalacturonate [21, 22]. *Dickeya* also produces low amount of polygalacturonases generating saturated oligo-galacturonates (pehN, pehVWX, pehK) [23], |24]. *D. dadantii* can utilize pectin as its sole carbon and energy source (Figure 1) [21]. The catabolic pathway of pectins (pectinolysis) has been characterized at the genetic and biochemical level. These studies have revealed that KDG, a catabolite product of pectin, plays a key role in pectinolysis by inactivating KdgR, the main repressor of this catabolic pathway [25-27]. The positive feedback loop between the extracellular pectin degradation by Pels and Pehs and the intracellular inactivation of KdgR by the metabolite KDG has been modeled [7, 28]. Refining this type of model requires a precise determination of the concentration of KDG present in the bacteria's growth environment. The conventional technique to determine the concentration of KDG so far is thiobarbituric assay [29]. Detection of KDG can be carried on by cellulose thin-layer chromatography by spraying with either periodate-thiobarbituric acid or *o*-phenylenediamine [30]. These methods are not very accurate for biological application since they are not very specific to KDG alone, but rather quantify DKI, DKII and KDG indistinctly. Here, we present a new method to quantify this metabolite using HPLC. The novel technique presented here allows quantifying KDG with increased precision and specificity with respect to functionally related compounds, as required for further regulatory models.

MATERIALS AND METHODS

Chemicals

KDG (95%), OPD (O-phenylenediamine), HPLC grade Trifluoroacetic acid (TFA) were from Sigma Aldrich (France). Partially purified DKI and DKII (90%) was a gift by Guy Condemine. Optima LC-MS acetonitrile (ACN) was from Fisher UK. Polygalacturonate dipecta (PGA) was from Agdia laboratory (France). All HPLC elutants, standards and assays were prepared using MilliQ pure water with a specific resistance of at least 18 mΩ.

Bacterial strains and culture conditions

Dickeya dadantii (strain 3937) and its derived *kduI*, *kduD* and *kdgK* mutants were used for all the experiments [21] (Table 1). Bacteria were grown at 30°C in M63 minimal medium supplemented with a carbon source: glucose (0.2% w/v) alone or in addition with PGA (0.4% w/v). Cells were harvested in the late exponential growth phase when the bacterial population has reached its maximum.

Sample collection
Bacteria were separated from the medium by centrifugation at 5000 *g* for 8 minutes. Cells and cultures medium samples were freeze dried with liquid nitrogen and stored separately at -80°C. Bacterial pellet was resuspended with a 10 ml cold solution of 2.5 % (v/v) TFA, 50% (v/v) acetonitrile. The dissolved lysed pellet was maintained at -80°C for 15 minutes and thawed at room temperature. The solution was then centrifuged at 20000 *g* for 5 minutes. The clear supernatant containing the metabolites (10 ml) was separated from the pellet and then lyophilized overnight. The dry powder was dissolved in 1 ml of 0.1 M HCl and centrifuged again to remove particles. Culture medium (10 ml) was lyophilized and re-suspended in 1 ml 0.1 M HCl.

Sample derivatization
200 µL of sample was incubated for 60 minutes at 60°C with 20 µL solution of 100 mM OPD prepared in 0.1 M HCl with agitation in an Eppendorf thermomixer as described previously [35], (Figure S1). Samples turn yellowish. Appropriate amounts of the metabolites-adduct were then injected into HPLC for analysis.

HPLC system and gradient elution
The HPLC system consisted of an Alliance HT Waters 2795 separation module and detection was performed with a Waters 2996 photodiode array detector interconnected with a fluorimeter SFM25 (Kontron). The OPD-adducts to be determined were passed through a 4.6x250-mm Uptisphere 5 µM HDOC18 column (Interchim) at 35°C and a flow rate of 1 mL/min. The elution protocol employed linear gradient as follow: initial 0 to 5 min, 100%A; 5 to 12 min, 75%A, 25%B; 12 to 20 min, 75%A, 25%B; 20 min to 30 min 75%A, 25% B to 100 %B; 30 min to 35 min, 100%B, then, 35 min to 37 min 100%B to 100%A and re-equilibrated for 3 min in 100%A before a new run. Solvent A consisted of 0.005% trifluoroacetic acid (TFA) and solvent B contained 60% acetonitrile. The OPD adduct(s) was detected using a photodiode array detector (Waters 2996) set up at 338 nm, and with a SFM25 fluorimeter (Kontron) equipped with a 8 µL flow cell. Emitted fluorescence of the OPD adduct(s) was measured with an excitation wavelength of 338 nm and an emission wavelength of 414 nm (slit sizes, 15 and 10 nm respectively, and lamp power of 400 W). Quantification of metabolites was carried out by measuring peak areas using the chromatography data system Mass-Link software and assigned intensity to the peak (Waters).

RESULTS AND DISCUSSION

The derivatization and subsequent HPLC procedure described here represents a successful improvement over the thiobarbituric method developed previously [24]. We used OPD as a probe for the carbonyl groups of metabolites such as KDG, DKI and DKII (Figure S1). Figure 2 shows a representative chromatogram of KDG standard solution. The standard KDG has a retention time of around 16 minutes. From the estimated area of the respective peaks, the fluorescence intensity is approximately 2500-fold more sensitive than the UV absorbance intensity. Thus, fluorescence is more accurate for the resolution and quantitation of the metabolite of interest. The ODP adduct of the carbonyl group of KDG is characterized by a typical spectrum with a maximum absorption in the limit of the visible range at 338 nm. Upon excitation at this wavelength, a maximum emission is measured at 414 nm (Figure 3A and B).

The unsaturated digalacturonate pathway has DKI and DKII as intermediate metabolites before the formation of KDG (Figure 1). The properties of derivatization based on the presence of a carbonyl group led us to hypothesize that these metabolites would be derivatized also in our experimental conditions. We performed the same procedure of analysis starting from a partially pure DKI/DKII mixture (obtained by purification from *kduI/kduD* mutants, G. Condemine, personal communication). The closeness of the structures of DKI and DKII makes it difficult to differentiate them. Consistently, the corresponding chromatogram reveals a major single peak with a retention time of about 21 minutes for these compounds (Figure 4). We hypothesized that the signature spectrum of the KDG molecule would be comparable to that of DKI or DKII since the molecular structures are closely similar (see Figure 1). Only the predominant peak present in the absorbance chromatogram (Figure 4A) shows the typical spectrum signature of KDG as described in Figure 3A. We therefore optimized the gradient to discriminate the KDG from other similar structured metabolites produced in the media culture like DKI and DKII, allowing quantification from the intensity of a single specific peak (Fig. 2).

Linearity

Linearity of the method was confirmed by preparing KDG standard curves for the analytical range of 0-5 nanomoles (Fig. 5). Statistical analysis using Least Square Regression (LSR) indicated excellent linearity in the mentioned range, with a correlation of $R^2 \geq 0.993$ for all standard curves with fluorescence detection and $R^2 > 0.988$ for the UV detection. The slopes of the calibration curves were 0.0026 and 6.64 for absorbance and fluorescence intensity detection, respectively (Figure 5). From the respective slopes, the fluorescence intensity detection is more sensitive than the absorbance intensity detection, with a ratio of around 2500-fold.

Limit of Detection and Limit of Quantification

The range of application of the method was verified by analyzing replicate samples containing the metabolite at various concentrations. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated by the linear regression method: the LOD is given by $3S_a/b$ and LOQ is given by $10S_a/b$, where S_a is the standard error of the intercept and b is the slope of the calibration curve [36]. Based on these equations for absorbance and fluorescence detection (Fig. 5), the calculated LOD values were found to be 0.093 nanomoles and 0.077 nanomoles respectively, and LOQ values were found to be 0.28 nanomoles and 0.23 nanomoles respectively, in our experimental condition settings.

Biological Sample Analysis

In order to test the method for KDG quantification, we used the *Dickeya dadantii* wild type strain 3937 and its *kduI*, *kduD* and *kdgK* mutant derivatives. Bacteria were grown on a glucose minimal medium supplemented or not with polygalacturonate (PGA), and samples were collected after 10 hours of growth, corresponding to the transition to stationary phase. The characteristic peak of KDG appeared only in the presence of addition of PGA in the medium, and was absent in cultures performed in glucose, as expected. Figure 6 depicts the chromatogram for the wild type bacterial samples with and without PGA in the isocratic phase of the gradient to amplify the image resolution of KDG-OPD adduct molecule. The full chromatogram for the wild type strain is depicted (Figure S2). KDG is eluted at around 16 min in a symmetrical peak while

DKI and DKII accumulated in the *kduI*, and *kduD* mutants, respectively at 21 min (Figure S3 and S4). As expected, in the *kdgK* mutant, a unique peak at 16 min is highlighted in presence of polygalacturonate in the medium (Figure S5).

Quantitation of metabolites

The KDG concentration was quantified in the wild type *D. dadantii* strain and the three mutant derivatives (*kduI*, *kduD* and *kdgK)* to study the formation of intracellular concentration of KDG (Figure 7). The intracellular quantification was carried out based on the assumption of a cell volume to be 3.6 $\mu L.mL^{-1}.OD^{-1}$ where optical density (OD) is measured at 600 nm [37]. In the medium supplemented with a PGA source the maximum quantification of KDG is observed in the *kdgK* mutant, as expected since it accumulates the KDG metabolite (up to 233 mM). The *kduI* and *kduD* mutants accumulated KDG at higher level than wild type cells (10- to 30-times respectively), due to the increased catabolism of saturated galacturonate in the hexuronate pathway. The Pel enzymatic activities assayed in the mutants also are consistent with the elevated levels of KDG (Table S1).

DKI and DKII could not be further differentiated on the chromatogram due to their structural similarity and unavailability of pure DKI and DKII compounds. We quantified DKI and DKII based on the KDG calibration curve considering that the absorbance and fluorescence slopes would be similar. In *kduI* mutants DKI is not converted into DKII, and its concentration was quantified around 15mM. In the *kduD* mutant the combined concentration of DKI+DKII was 31mM. The *kdgK* mutant had no detectable DKI or DKII molecules.

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Conclusion

To demonstrate that our technique is specific for KDG alone, we showed that the retention time for the derivatives DKI/DKII is 21 minutes whereas the retention time for KDG is around 16 minutes. Nonetheless, this method is solid enough to eliminate the suspicion of cross derivatization between KDG and DKI/DKII. We do not consider galacturonate pathway since the similarity in the structure of compounds are not significant for the possibility of cross derivatization.

The fluorescent technique was 2500-fold more sensitive over the absorbance detection. The results also indicate the higher reliability of the fluorescence technique over the absorbance detection method since it has a lower coefficient of variation. Nonetheless, the combination of the two techniques provide further validation of the results and stronger quantification.

Thus, we describe a robust method for the quantification of the metabolite KDG from biological samples. This method will help in the analysis and quantitative modelling of gene regulatory networks where KDG is known to have any effect [7, 28]. Moreover, quantifying metabolite KDG flux is critical for biofuel production during pectin and alginate degradation or for other high value products derived from these two polymers [20]. There is also potential scope for the industrial application of this technique in metabolic engineering of compounds like Vitamin C from KDG [38].

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Table 1: *Dickeya dadantii* strains used in the study

Strain	Genotype	Reference
3937	Wild Type	[31]
A2395	*kduI::kan*	[32]
A797	*kduD::kan*	[33]
A3999	*kdgK::kan*	[34]

Legends to Figures

Figure 1: Schematic metabolic pathways leading to KDG (2-Keto-3-deoxy-gluconate) production from pectin and alginate degradation.

Pectin degradation involved pectate lyases and polygalacturonases that cleaved the polygalacturonate, the demethylated pectin, into unsaturated and saturated digalacturonate, respectively. Inside the bacteria, these coumpounds are converted by Ogl enzyme into galacturonate and unsaturated galacturonate (for saturated digalacturonate) and into two molecules of unsaturated galacturonate (for unsaturated digalacturonate). KdgF converts unsaturated galacturonate into DKI. KduI enzyme then converts DKI to DKII and KduD subsequently converts DKII to KDG, 2-Keto-3-deoxy-gluconate. This compound is then phosphorylated by a specific kinase KdgK and finally cleaves by KdgA to yield pyruvate and phospho-glycerate. The galacturonate is degraded to KDG by the enzymes UxaC, UxaB, UxaA in the hexuronate pathway.

Alginate degradation involved alginate lyases that cleaved the corresponding polymer into unsaturated dimannuronate and unsaturated diguluronate. Inside the bacteria, these compounds are converted by oligouronide lyase Oal into unsaturated mannuronate and unsaturated guluronate monomers. These 4,5-unsaturated monouronates are converted to DEH (4-deoxy-L-erythro-5-hexoseulose uronate) by the enzyme KdgF. DEH is then reduced into KDG by the Sdr enzyme.

Enzymes stand for: Pels, pectate lyases; Pehs, polygalacturonases; Ogl, oligogalacturonate lyase; KdgF, responsible for linearisation of 4,5-unsaturated monouronates to linear ketonized forms; KduI, 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase; KduD, 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase; KdgK, 2-dehydro-3-deoxygluconokinase; KdgA, 2-dehydro-3-deoxy-phosphogluconate aldolase; UxaC, D-galacturonate isomerase; UxaB, Altronate oxidoreductase; UxaA, Altronate dehydratase; Agls, alginate lyases; Oal, oligouronide lyase; Sdr, 4-deoxy-L-erythro-5-hexoseulose uronate reductase.

Figure 2 : Chromatogram for the detection of 1 nanomole of standard KDG-OPD molecule adduct. A) Absorbance and B) Fluorescence detection.

Figure 3 : A) Characteristic of the absorbance spectrum of 1 nanomole of the KDG-OPD molecule adduct derived from the peak previously isolated by HPLC. B) Excitation and emission peaks of the purified KDG-OPD molecule adduct purified above. Spectra were performed in the elution buffer using a SFM25 spectrofluorimeter (Kontron).

Figure 4 : Chromatogram of the partially pure DKI or DKII samples. A) Absorbance and B) Fluorescence detections. Inserts showed spectra of the DKI or DKII –OPD, and of the minor unknown metabolite. Only, the peak eluted at 21 min, based on the similarity of the structure with KDG showed the typical spectrum as reported in figure 3. It should be noticed that the unknown metabolite adduct is characterized by a greater yield in fluorescence emission upon excitation at 338 nm.

Figure 5: Standard curves for the pure KDG metabolite. A) Absorbance intensity at 338 nm and B) Fluorescence intensity at 414 nm upon excitation at 338 nm. Inserts reported the value of the linear slope (a) with R^2 being the regression coefficient. As noticed, the fluorescence intensity is more accurate than the absorbance intensity detection in our experimental set-up of the fluorimeter (2500-fold more sensitive).

Figure 6: Chromatogram of sample from wild-type bacterial cells grown in the presence (continuous black line) and absence (gray dotted line) of pectin. The chromatogram is in the isocratic phase of the gradient to amplify the image for resolution of KDG-OPD adduct molecule. A) Absorbance intensity at 338 nm and B) Fluorescence intensity at 414 nm.

Figure 7: Logarithmic representation of KDG concentrations measured in wild type *Dickeya dadantii* and *kduI*, *kduD*, *kdgK* mutants affected from steps of the pathway of unsaturated digalacturonate (Figure 1). Error bars indicate the standard error observed in experimental quantifications over biological triplicates.

Table S1 : Pel activity in WT, *kdgK, kduI, kduD* mutants

Strain	Growth medium	Pel activity
WT	Glucose	0.3
	Glucose +PGA	7.4
kdgK	Glucose	0.5
	Glucose +PGA	37.0
kduD	Glucose	0.2
	Glucose +PGA	15.0
kduI	Glucose	0.2
	Glucose +PGA	12.0

Representative pectate lyase production of *Dickeya dadantii* wild type strain and mutant strain grown in minimal medium supplemented with glucose or glucose + polygalacturonate. Samples were taken in late stationary phase. Pel specific activity is expressed as µmol of unsaturated product liberated per min per mg of bacterial dry weight. These values were reported from literature and measures performed in this study.

Figure 1

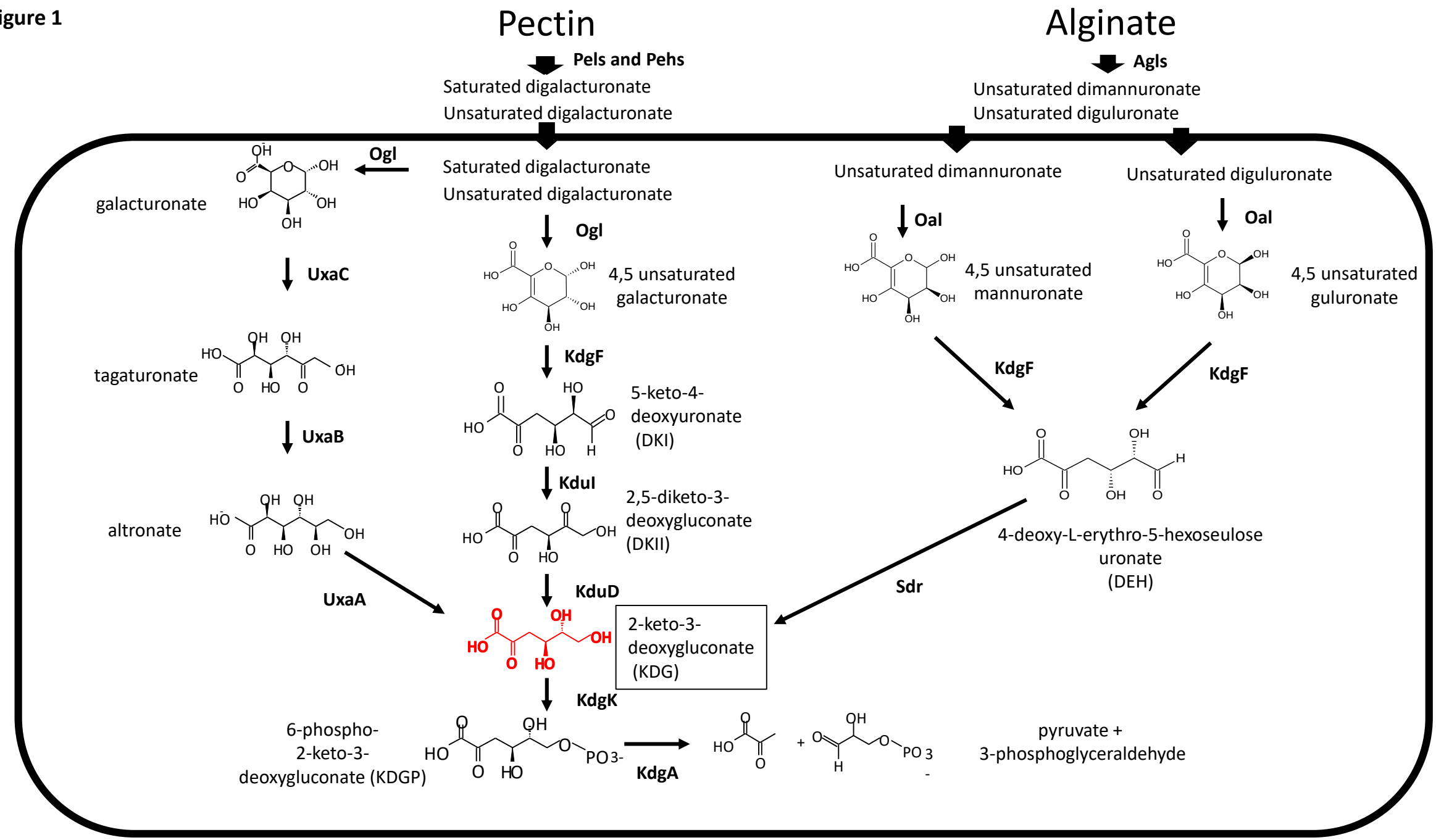

Pectin
Pels and Pehs
Saturated digalacturonate
Unsaturated digalacturonate
Alginate
Agls
Unsaturated dimannuronate
Unsaturated diguluronate
Ogl
galacturonate
UxaC
tagaturonate
UxaB
altronate
UxaA
Saturated digalacturonate
Unsaturated digalacturonate
Ogl
4,5 unsaturated galacturonate
KdgF
5-keto-4-deoxyuronate (DKI)
KduI
2,5-diketo-3-deoxygluconate (DKII)
KduD
2-keto-3-deoxygluconate (KDG)
KdgK
6-phospho-2-keto-3-deoxygluconate (KDGP)
KdgA
pyruvate + 3-phosphoglyceraldehyde
Unsaturated dimannuronate
Oal
4,5 unsaturated mannuronate
KdgF
Unsaturated diguluronate
Oal
4,5 unsaturated guluronate
KdgF
4-deoxy-L-erythro-5-hexoseulose uronate (DEH)
Sdr

Figure 2

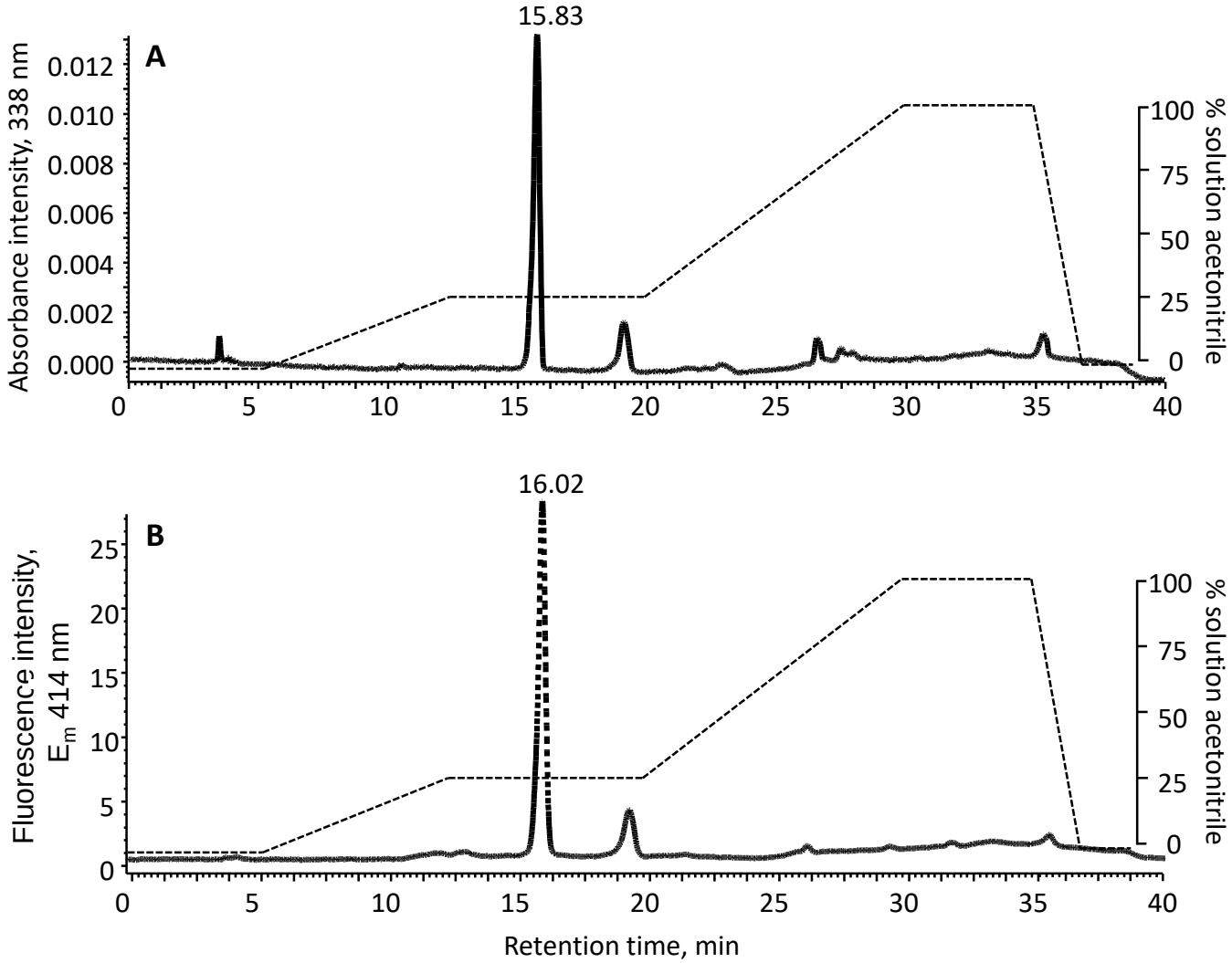

15.83
A
Absorbance intensity, 338 nm
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
5
10
15
20
25
30
35
40
100
75
50
25
0
% solution acetonitrile
16.02
B
Fluorescence intensity, E_m 414 nm
25
20
15
10
5
0
Retention time, min

Figure 3

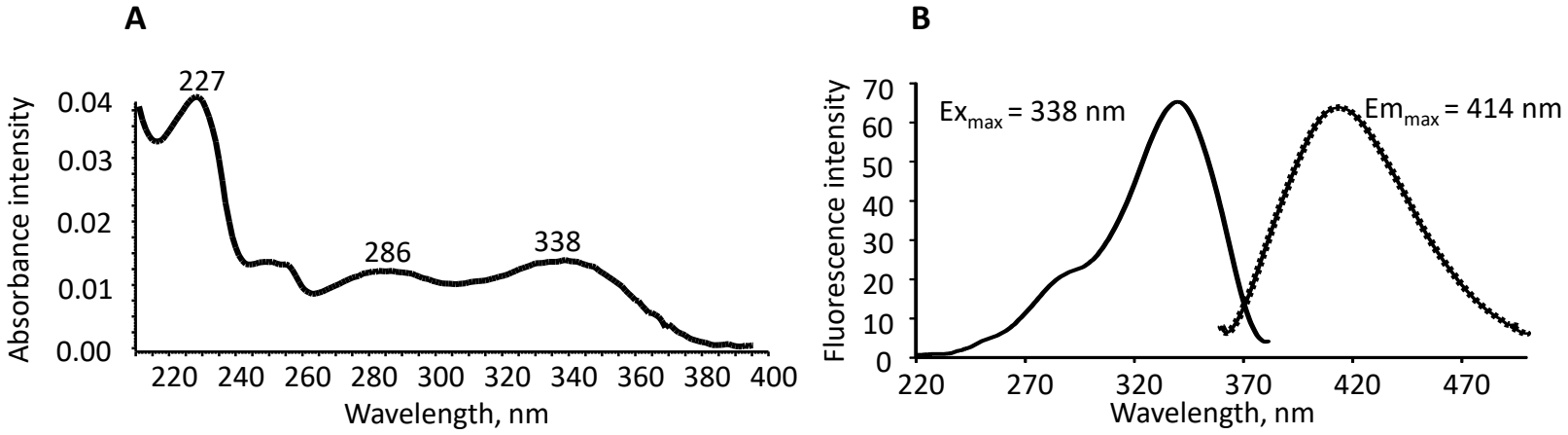

A
227
286
338
Absorbance intensity
0.04
0.03
0.02
0.01
0.00
220 240 260 280 300 320 340 360 380 400
Wavelength, nm
B
Ex$_{max}$ = 338 nm
Em$_{max}$ = 414 nm
Fluorescence intensity
70
60
50
40
30
20
10
0
220 270 320 370 420 470
Wavelength, nm

Figure 4

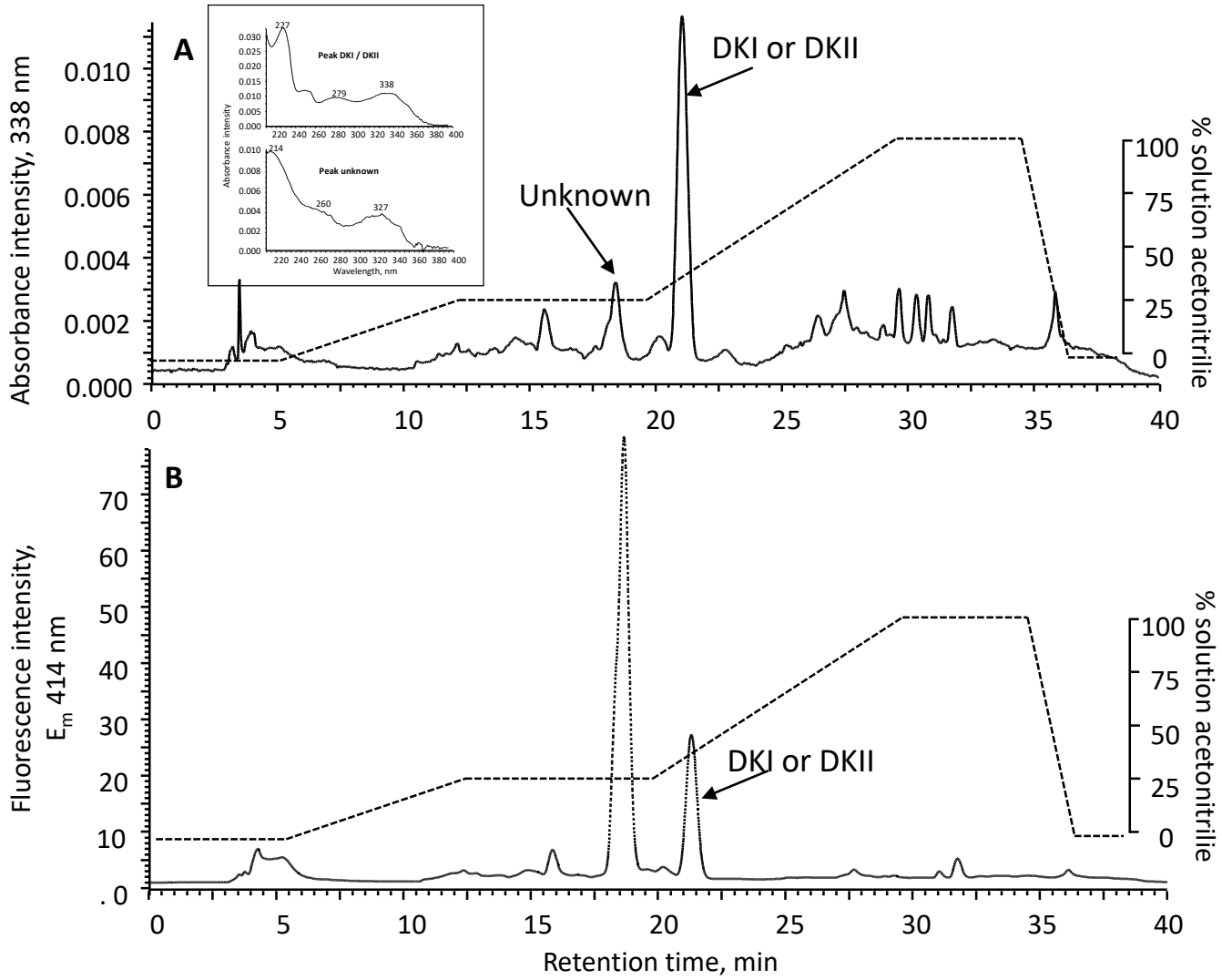
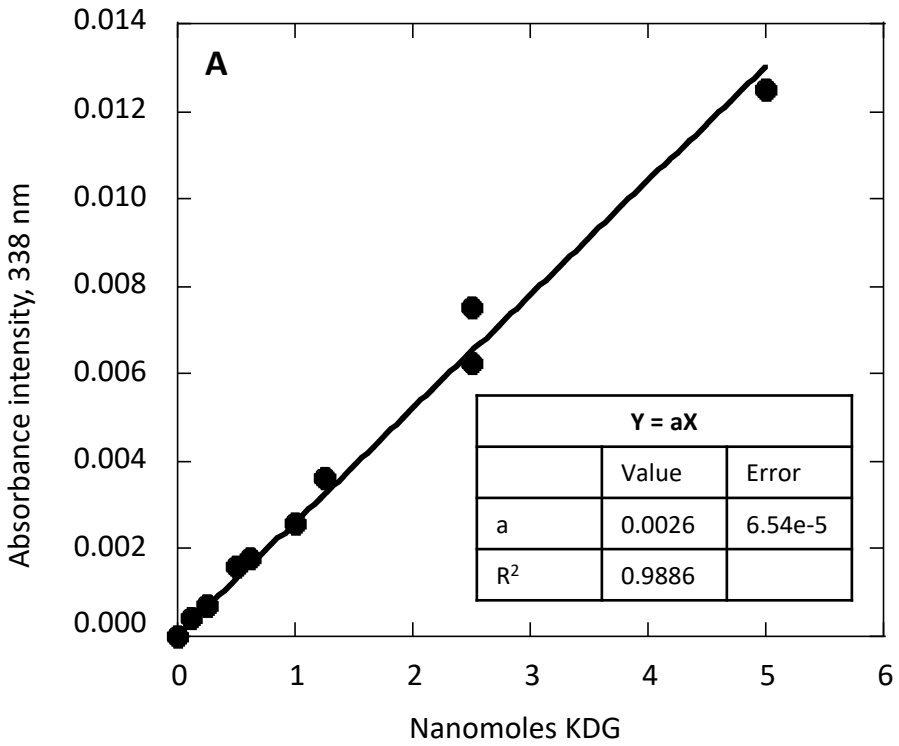

A
Absorbance intensity, 338 nm
0.010
0.008
0.006
0.004
0.002
0.000
Peak DKI / DKII
227
279
338
Peak unknown
214
260
327
Absorbance intensity
Wavelength, nm
Unknown
DKI or DKII
% solution acetonitrilie
100
75
50
25
0
0
5
10
15
20
25
30
35
40
B
Fluorescence intensity,
E_m 414 nm
70
60
50
40
30
20
10
. 0
DKI or DKII
% solution acetonitrilie
Retention time, min

Figure 5


A
Absorbance intensity, 338 nm
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
1
2
3
4
5
6
Nanomoles KDG
Y = aX
Value
Error
a
0.0026
6.54e-5
R2
0.9886

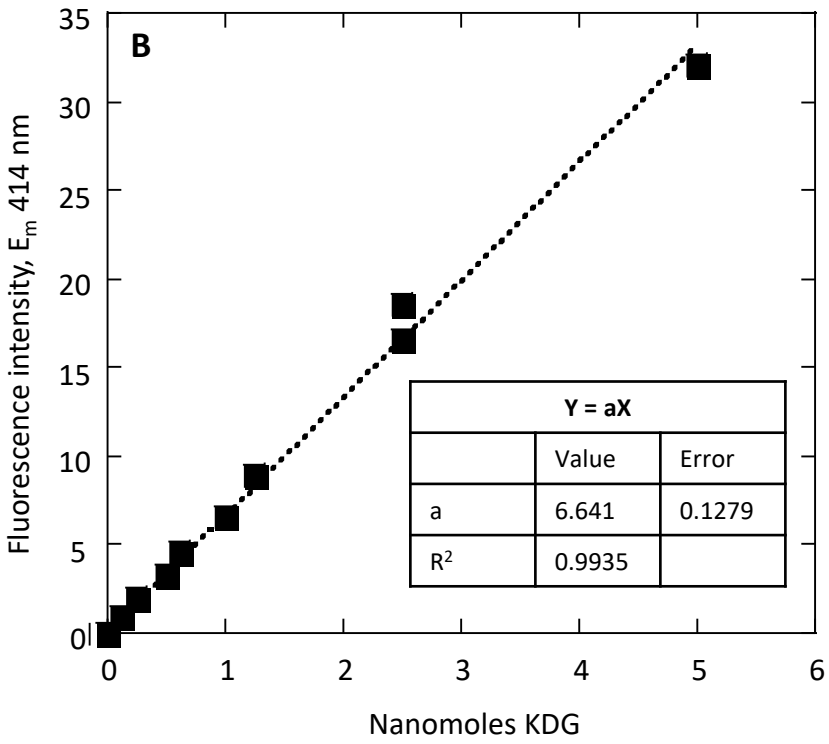

B
Fluorescence intensity, Em 414 nm
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Nanomoles KDG
Y = aX
Value
Error
a
6.641
0.1279
R2
0.9935

Figure 6

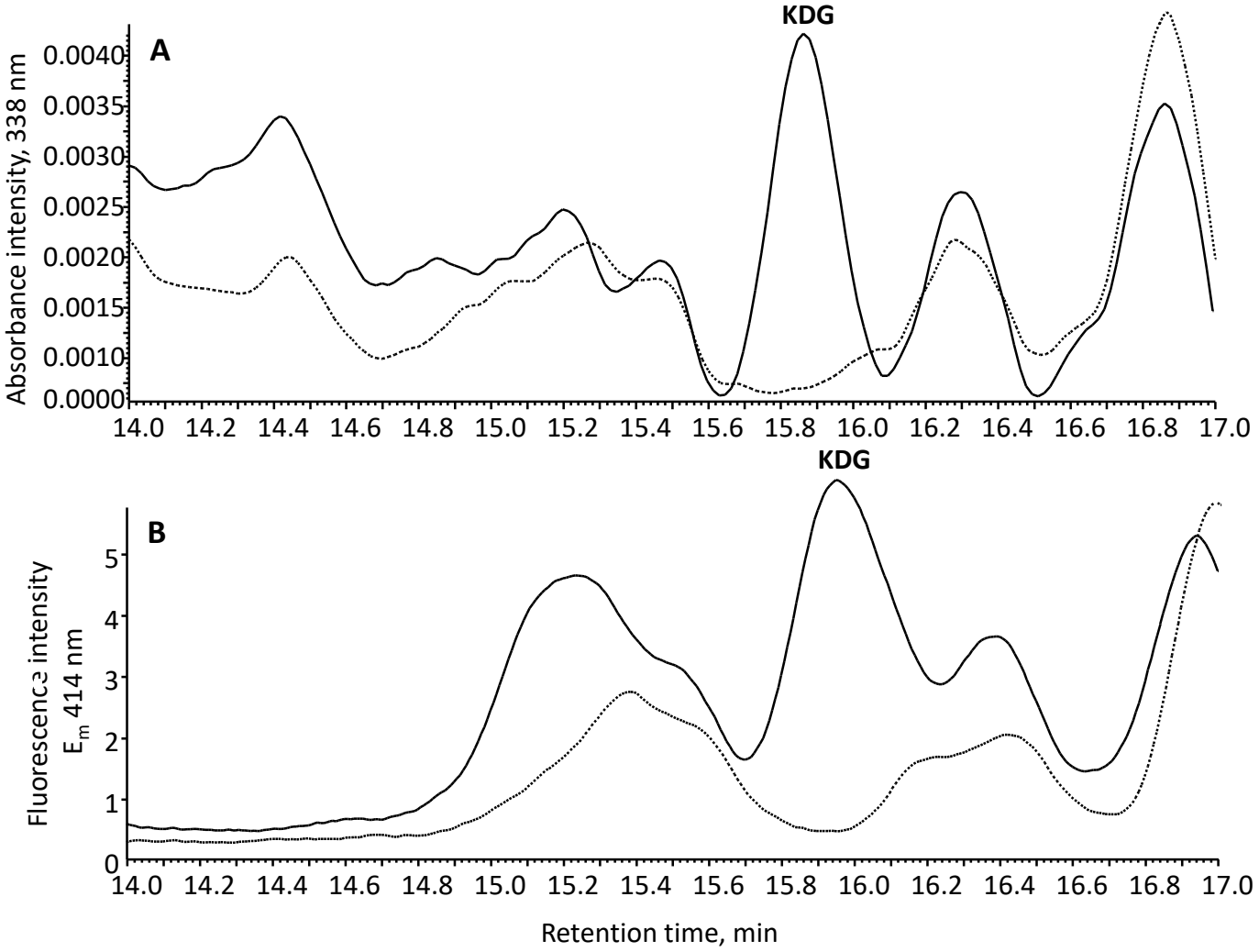

KDG
A
Absorbance intensity, 338 nm
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0000
14.0 14.2 14.4 14.6 14.8 15.0 15.2 15.4 15.6 15.8 16.0 16.2 16.4 16.6 16.8 17.0
KDG
B
Fluorescence intensity
E_m 414 nm
5
4
3
2
1
0
14.0 14.2 14.4 14.6 14.8 15.0 15.2 15.4 15.6 15.8 16.0 16.2 16.4 16.6 16.8 17.0
Retention time, min

Figure 7

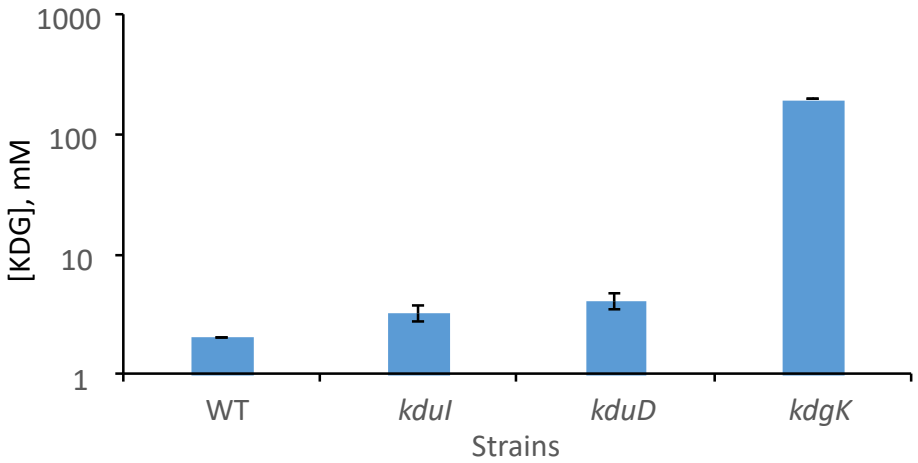


Figure S1: OPD derivatization of carbonyl groups of metabolites such as KDG, DKI, DKII

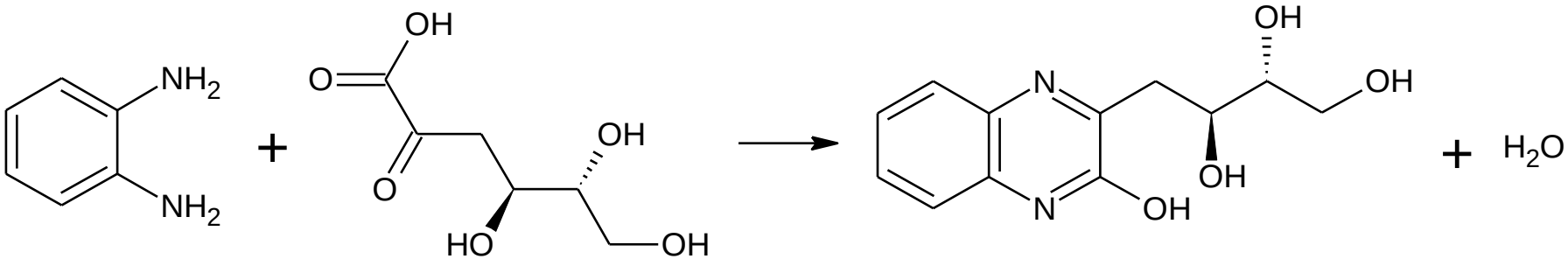


Figure S2: Chromatogram of sample from the wild type strain

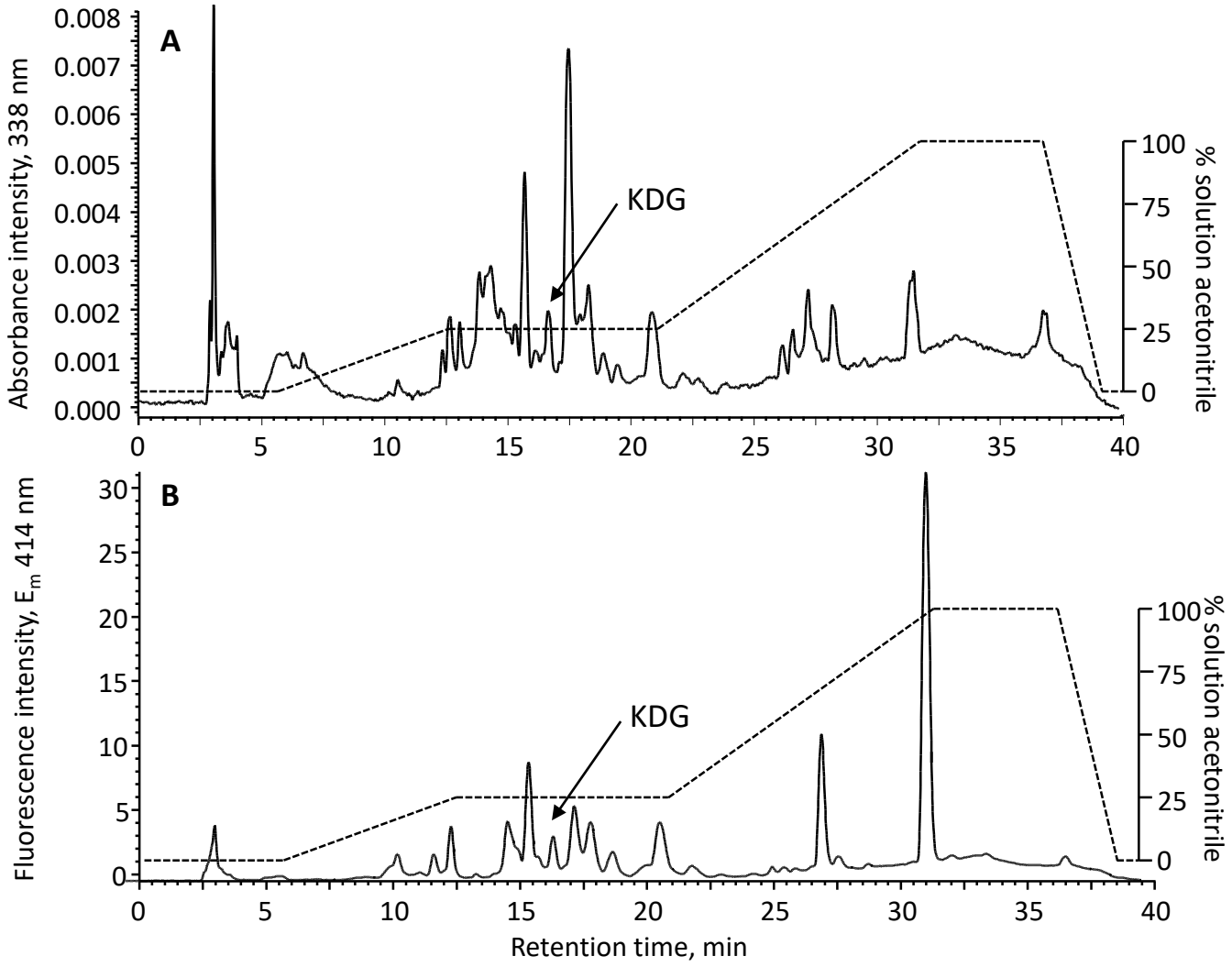


Figure S3: Chromatogram of sample from *kduI* mutant

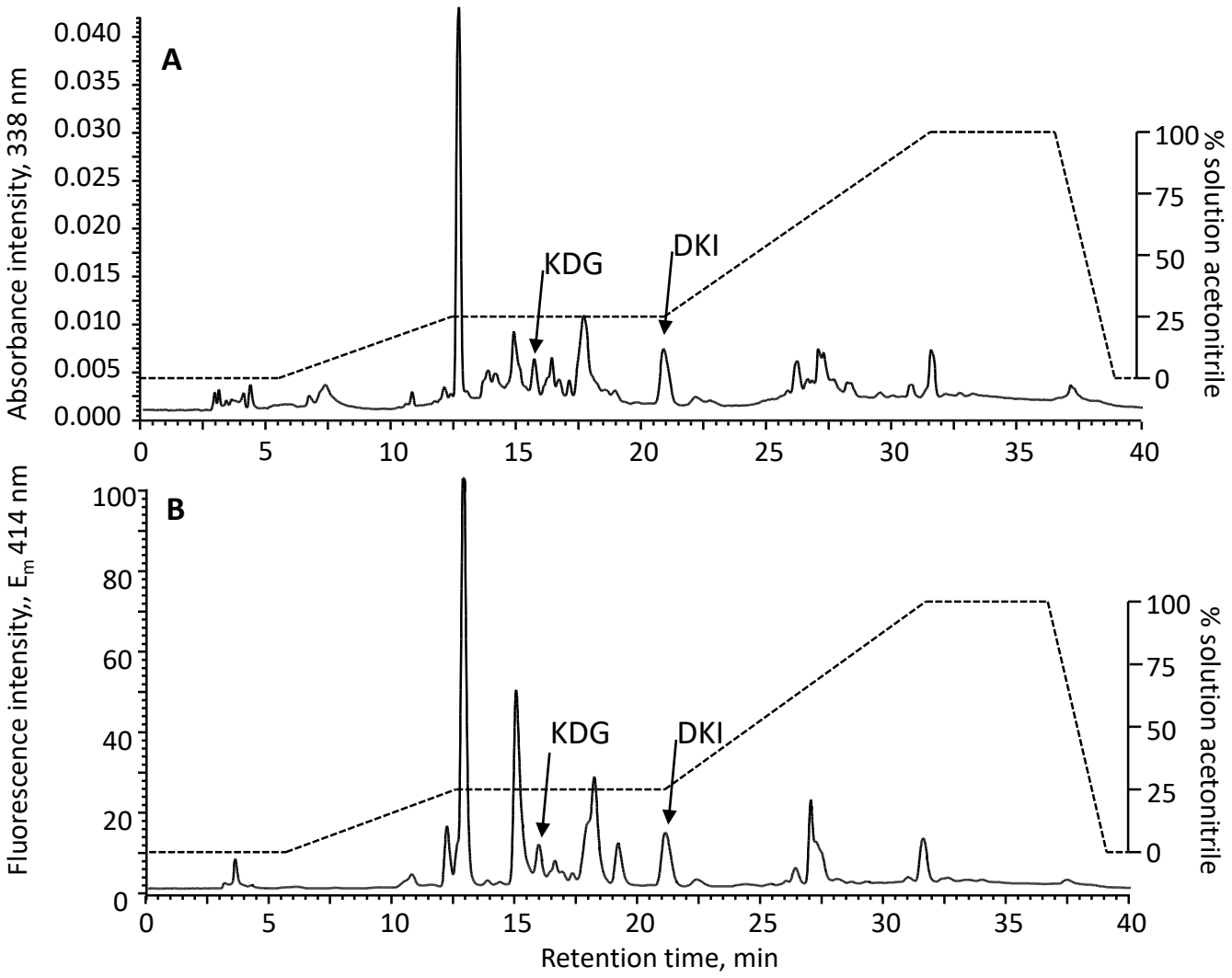


Figure S4: Chromatogram of sample from the *kduD* mutant

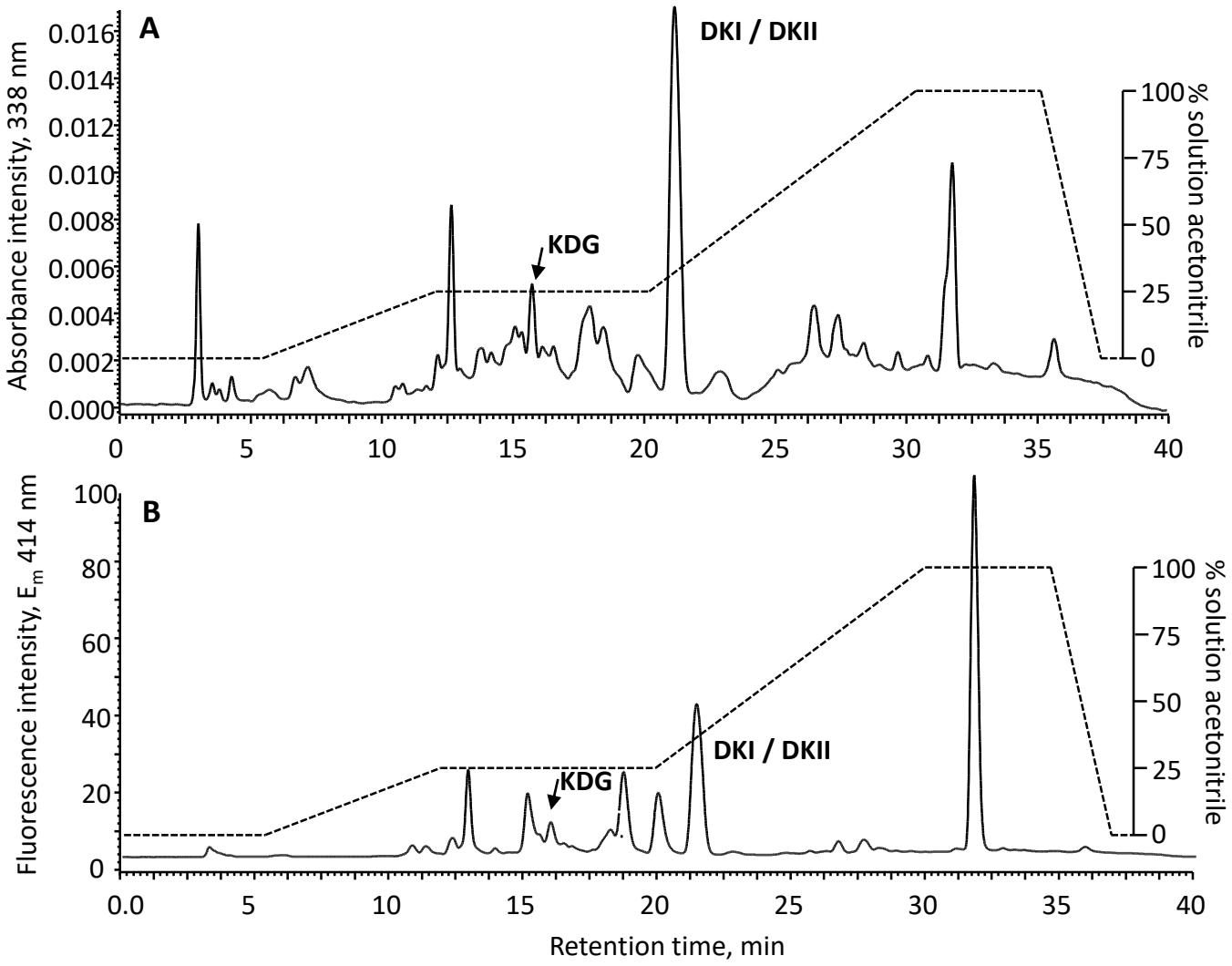
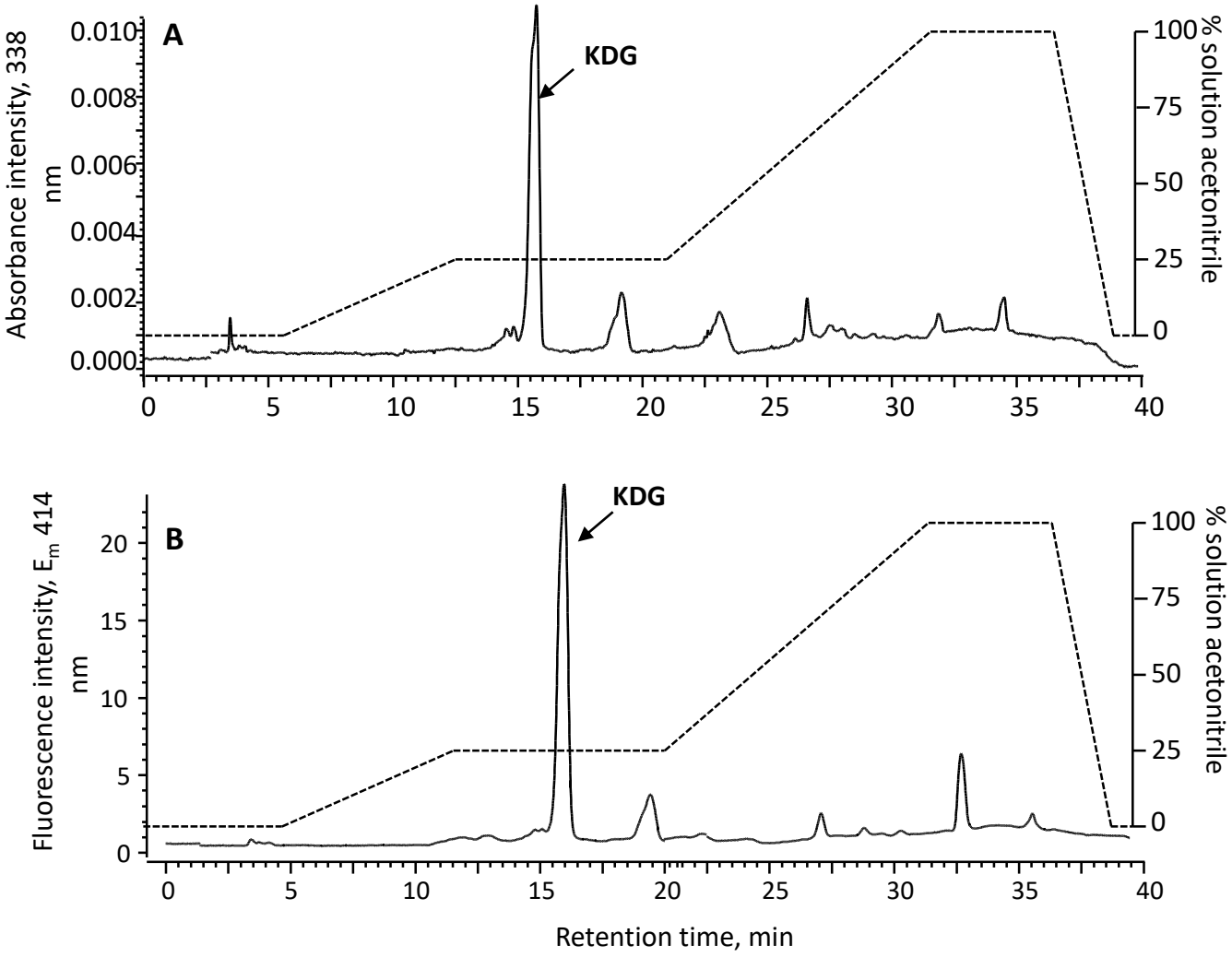



Figure S5: Chromatogram of sample from the *kdgK* mutant