Research Article

Untargeted metabolite analysis of healthy and Huanglongbing-infected orange leaves by CE-DAD

Huanglongbing (HLB) is considered the most destructive bacterial citrus disease worldwide. Early detection of HLB is crucial for minimizing its spread. CE was used for the discovery of potential biomarkers for HLB. Optimization of extraction and separation allowed resolving 24 compounds of which 6 were present in significantly higher ($p<0.05$) concentrations in HLB-infected samples collected monthly for 6 months during the 2007–2008 season. Three of these compounds were identified by mobility and UV spectra as hesperidin, naringenin, and quercetin with mean increase in concentration of 154, 555, and 467%, respectively, above that in healthy leaves. Results support the potential of CE-DAD for untargeted plant metabolomic analysis. CZE, NACE, and MEEKC were compared for metabolic differentiation of healthy and HLB-infected citrus leaves. CZE in a semi-aqueous BGE solution consisting of 8.5 mM of sodium borate (pH 9.3), 15% ACN, and 9% 1-butanol yielded the best peak separation with detection at 190 nm.

Keywords:
Biomarkers / CE / Huanglongbing / Metabolomics / Plant diseases

1 Introduction

Citrus Huanglongbing (HLB) is the most significant threat of the citrus industry worldwide [1]. It originated in Asia and first detected in the Americas in 2004 (Brazil), reaching the United States in 2005 [2]. The causal agent is Candidatus Liberibacter, a gram-negative bacteria not yet cultured to Koch’s postulates. Present methods for its quantification based on real-time PCR are currently being optimized [3]. The disease is transmitted through a psyllid (Diaphorina citri) vector that feeds on citrus and other tropical and sub-tropical plants making vector eradication impossible. Symptoms of the disease include marked yellow regions on leaves and poor quality, small, inedible, and misshapen fruit [4]. HLB has had serious damaging impact in the citrus industry in many Asian countries, wiped out a great number of trees in Brazil, and as of 2008, has seriously affected groves in 26 Florida counties making Candidatus Liberibacter the most dangerous citrus pathogen in the world [5]. Since the appearance of HLB, many efforts have been directed toward controlling this disease including frequent pesticide spray to reduce psyllid population, the use of pheromone traps, and psyllid repellents. Early detection of HLB-infected trees is crucial to managing HLB’s spread by reducing inoculums through tree elimination.

Currently, PCR is the only approved method for diagnosis of HLB; however, this is an expensive, laborious, and time-consuming alternative that does not allow in-field analysis. Other limitations such as the low concentration and uneven distribution of the bacteria in the tree [6] make PCR detection very difficult, especially at early stages. Research on new methods such as the isothermal and chimeric primer-initiated amplification of nucleic acids combined with cycling probe technology [7], application of nested PCR [8], comparisons of primers for PCR and nested PCR [9], along with the improvement of DNA isolation for conventional PCR [10] has been reported. However, PCR amplification of the bacteria is very weak during spring and summer seasons [11], increasing the probability of obtaining false negatives during these periods. Therefore, methods that do not rely on the presence of the bacteria in the sample are sought as more reliable throughout the year. To the best of our knowledge, only two non-PCR methods have been comprehensively researched. One relies on the presence of excessive amounts of starch [12], whereas the other on overproduction of gentisic acid [13] in infected trees. The disadvantages of these methods are that they are not HLB-specific, because excessive amounts of starch are noticed with other stress situations such as girdling [14] and gentisic acid overproduction is caused by other infections in several...
plants [15]. It can be hypothesized that using a group of compounds as “fingerprint” for HLB has greater potential for specificity than a single compound because different specific concentration ratios and specific metabolite patterns may result for a particular infection. Also, the identification of new possible HLB biomarkers will provide a new basis for performing selectivity analyses. Metabolomics allows the detection of a group of possible biomarkers in a given system and has been shown to be a powerful tool for biomarker discovery in plant extracts [16]. Initial stages for biomarker discovery, fingerprinting, and footprinting must include all possible metabolites present in a system, so untargeted metabolomics analyses are desirable [17, 18]. Several forms of CE have been successfully used in targeted analysis [19–25]. Untargeted metabolomics studies are very limited [18], and in most cases involve the use of CE coupled to ESI-MS system [17]. However, BGE formulations capable of being used in an ESI system are limited to low salts, and volatile components that do not necessarily provide the best separation efficiency [18]. Another limitation is the extra cost of the ESI-MS system.

CE-DAD is a powerful and inexpensive separation tool. It has been successfully applied in untargeted analysis in mice [26]. García-Pérez et al. [27] demonstrated the effectiveness of CE-DAD for non-targeted metabolic differentiation of Schistosoma mansoni infection in mice. Recently, untargeted CE-DAD analysis was successfully used for fingerprinting Siraitia grosvenorii for quality monitoring purposes [28]. However, to the best of our knowledge, there is no report on untargeted metabolomics analysis for fingerprinting of plant diseases using CE-DAD. The objective of this research was to use CE-DAD for untargeted analysis of plant metabolites and develop a CE-DAD method for characterization of possible citrus HLB biomarkers.

2 Materials and methods

2.1 Reagents

HPLC-grade reagents (methanol, chloroform, and ACN), sodium tetaborate decahydrate, 1- butanol, ethanolamid, sodium acetate, sodium phosphate, hexane, SDS, naringenin, narirutin, hesperidin, tangeritin, synephrine, quercetin, gentisic acid, and ferulic acid were purchased from Fisher Scientific (Pittsburg, PA). Deionized, ultrafiltered water was used for all experiments.

2.2 Equipment and software

The water bath used for all the experiments was an Isotemp 3016S from Fisher Scientific. The CE system model P/ACE MDQ with DAD, the data acquisition and analysis software Karat 32 version 5.0 was from Beckman Coulter (Fullerton, CA). The capillary was bare fused silica from Polymicro Technologies (Phoenix, AZ) 50 μm id, 56 cm total length (48 cm to the detector).

ANOVA was carried out using SAS 9.0 from SAS Institute (Cary, NC) and significant differences were reported at 95% confidence level.

2.3 Sampling and experimental design

Healthy and diseased leaves were sampled from “Valencia” orange trees of the same age and type of shoot (summer and spring shoots from 10-year-old trees) in a commercial grove in Plant City, FL. Sampling started 4 wk after symptoms were discovered. However, as of 2008, there was no way to estimate when the trees were initially infected by HLB. PCR analyses were outsourced to the Plant Pathology Laboratory at the University of Florida’s Citrus Research and Education Center in Lake Alfred, FL. At least six leaves from three different PCR-positive and PCR-negative trees were sampled monthly from November 2007 to April 2008 to assess changes in metabolite profiles caused by uncontrolled changes in climate and other seasonal stress. After using some of the samples for PCR and CE optimization experiments, 36 HLB-infected and 18 healthy leaves were individually run under CE-DAD optimized conditions and statistically compared. Samples were stored on dry ice during transportation (45 min), and then stored at −80°C until analyzed (~1 month).

2.4 Extraction, sample preparation, and CE conditions tested

Solvents with different polarities were tested in both healthy and diseased samples to maximize extraction of the compounds that showed significant differences between control and infected samples. Individual leaves (~0.45 g) were ground to a fine powder under liquid nitrogen. Solvent was added to reach a final concentration of 4% w/v of ground tissue. Pure water, methanol, and chloroform were tested as solvents for extraction. The combination methanol/water/chloroform (MWC) used by Gullberg et al. [29] was also tested in an 8:1:1 ratio because a variety of compounds with different polarities was expected in this type of sample. The mixture was sonicated on ice for 30 min in a sonicator model FS20H from Fisher Scientific. Three extraction time–temperature combinations were tested: 12 h at 4°C, 12 h at 0°C, and 2 h at 60°C in the temperature-controlled water bath. After extraction, samples were filtered using 0.45 μm nylon syringe filters and ferulic acid was added to a final concentration of 100 mg/L as internal standard (IS). Endogenous ferulic acid was not detected in either healthy or infected samples under tested conditions, and did not interfere with any peaks in the electropherograms. The effect of pH was tested by adjusting the aqueous borate solution with 1 N NaOH or 1 N HCl as needed to reach pH values of 10.91, 9.30, 8.08, 6.51, 5.20, and 3.81. Sodium
phosphate adjusted to pH 6.51 with 1 N HCl and sodium acetate adjusted to pH values of 5.20 and 3.81 with 1 N HCl were also tested as BGE to evaluate the influence of a buffering system at those low pH in which borate does not have buffering capacity. Phosphate and acetate buffers were prepared at half and three times the borate concentration, respectively, to keep ionic strength constant. Adjustment of pH was done before the addition of the organic BGE components. Organic modifiers tested were ACN in the 0–30% range and 1-butanol in the 0–15% range.

2.5 Selected extraction and CE analysis conditions

Of the extraction and CE analysis conditions tested, the following provided the best results. Extraction conditions required the use of MWC in an 8:1:1 ratio as the solvent, followed by 30 min sonication on ice, and 12 h extraction at required the use of MWC in an 8:1:1 ratio as the solvent, followed by 30 min sonication on ice, and 12 h extraction at

Extraction conditions

Initially, the use of MWC in an 8:1:1 ratio as the solvent, followed by 30 min sonication on ice, and 12 h extraction at room temperature was investigated. To evaluate the influence of the solvent, extraction with chloroform yielded nine peaks of the 24 detected by optimum CZE conditions. Methanol alone was not effective at extracting non-polar compounds. Typical electropherograms comparing the migration times and UV spectra of the peaks with those of pure standards run under the same conditions showed the lowest extraction efficiency because they each were only able to yield ten peaks. Based on migration time and UV spectra comparison, the same compounds and 14 others were extracted with pure methanol. However, methanol alone was not effective at extracting non-polar compounds. Typical electropherograms are shown in Fig. 1.

Chloroform extracts were run under MEEKC conditions. Extraction with chloroform yielded nine peaks of which five were not detected with water and methanol-based

Table 1. List of the 24 peaks and IS detected by optimum CZE conditions as described in Fig. 6

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Migration time (min)</th>
<th>Mobility (cm² V⁻¹ s⁻¹)</th>
<th>λ_max (nm)</th>
<th>Possible compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.01</td>
<td>19.17</td>
<td>227</td>
<td>Unknown</td>
</tr>
<tr>
<td>2</td>
<td>7.31</td>
<td>18.38</td>
<td>190, 234 low</td>
<td>Synephrine</td>
</tr>
<tr>
<td>3</td>
<td>7.51</td>
<td>17.89</td>
<td>190, 210, 330</td>
<td>Tangeritin</td>
</tr>
<tr>
<td>4</td>
<td>8.43</td>
<td>15.94</td>
<td>204, 280</td>
<td>Hesperidin</td>
</tr>
<tr>
<td>5</td>
<td>8.63</td>
<td>15.57</td>
<td>205, 286</td>
<td>Narirutin</td>
</tr>
<tr>
<td>6</td>
<td>9.41</td>
<td>14.28</td>
<td>190, 250, 350</td>
<td>Unknown</td>
</tr>
<tr>
<td>7</td>
<td>10.13</td>
<td>13.26</td>
<td>190, 210</td>
<td>Unknown</td>
</tr>
<tr>
<td>8</td>
<td>10.48</td>
<td>12.82</td>
<td>196, 310</td>
<td>Naringenin</td>
</tr>
<tr>
<td>9</td>
<td>10.73</td>
<td>12.52</td>
<td>190, 210, 230</td>
<td>Oxalic acid</td>
</tr>
<tr>
<td>10</td>
<td>11.06</td>
<td>12.15</td>
<td>230, 275</td>
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<td>11.47</td>
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</tr>
<tr>
<td>13</td>
<td>12.34</td>
<td>10.89</td>
<td>220, 276, 320</td>
<td>Quercetin</td>
</tr>
<tr>
<td>14</td>
<td>12.65</td>
<td>10.62</td>
<td>210, 300</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>15</td>
<td>13.39</td>
<td>10.03</td>
<td>200, 310</td>
<td>Gentisic acid</td>
</tr>
<tr>
<td>16</td>
<td>14.64</td>
<td>8.17</td>
<td>190, 220</td>
<td>Unknown</td>
</tr>
<tr>
<td>17</td>
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<td>8.08</td>
<td>227</td>
<td>Unknown</td>
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</tr>
<tr>
<td>24</td>
<td>24.75</td>
<td>5.43</td>
<td>234</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* a) These compounds showed a significant difference (p<0.05) between healthy and diseased samples.

3 Results and discussion

3.1 Extraction

Extraction with water at 4 and 60°C for 12 and 2 h, respectively, were compared with methanol and chloroform extracts at 0°C. Cold water (CW), hot water (HW), methanol, and MWC extracts were run under CZE conditions. HW and CW showed the lowest extraction efficiency because they each were only able to yield ten peaks. Based on migration time and UV spectra comparison, the same compounds and 14 others were extracted with pure methanol. However, methanol alone was not effective at extracting non-polar compounds. Typical electropherograms are shown in Fig. 1.

Chloroform extracts were run under MEEKC conditions. Extraction with chloroform yielded nine peaks of which five were not detected with water and methanol-based
extractions according to UV spectra matching. No significant difference ($p>0.05$) was found when comparing healthy and infected chloroform extracts. Therefore, pure chloroform as extraction solvent was not further considered. MWC extraction was selected for the remaining experiments because it showed the best extraction efficiency because the analytes detected included those obtained from water, methanol, and chloroform extracts alone based on UV spectra comparison.

### 3.2 CZE

Several CZE separation parameters were tested. Increasing pH values from 3.61 to 8.08 increased the analyte mobility as shown in Fig. 2. This is due to the increase in the EOF caused by greater ionization of silanol groups in the capillary wall at high pH [31]. In contrast, pH values of 9.30 and above decreased the mobility of most of analytes. This may be explained by the high flavonoids content in plant extracts, which usually have $pK_a$-values above 8.5 [32]. Therefore, the net negative charge at those pH values may cause attraction to the opposite pole. The use of phosphate as BGE was also tested at pH 6.51. Phosphate concentrations tested were half borate concentration in order to test under same ionic strength. In the same manner, acetate was also tested as BGE at pH values of 5.20 and 3.80. Acetate concentration was three times greater than borate concentration but of equal ionic strength. Both buffers yielded separation and peak intensity very similar to that obtained by borate BGE at the same pH. As shown in Fig. 2, sodium borate pH 9.30 resulted in the best resolution and therefore was chosen for further experiments. A similar effect was observed when changing the BGE concentration.

Sodium borate concentrations were tested from 5 to 75 mM. The best compromise between peak resolution and analysis time was with 8.5 mM sodium borate. High concentrations of sodium borate improved separation at the expense of the EOF, which decreased with increasing ionic strength as previously reported [33]. However, borate concentrations greater than 8.5 mM caused peak broadening and even disappearance of the late migrating peaks, possibly due to higher longitudinal diffusion and stronger interaction of analytes with the capillary at low EOF [34]. The use of ACN as an organic modifier in CZE and NACE has been widely studied [35, 36]. However, optimal ACN concentration depends on the type of analyte and BGE used. Increase in ACN concentrations in the range of 0–30% caused increments in the migration time as shown in Fig. 3.

EOF reduction by ACN in aqueous BGE has been reported before [35, 36]. According to Smoluchowski's equation, a decrease in permittivity and zeta potential will decrease EOF:

$$\mu_{eo} = \frac{\zeta e}{\eta}$$

where $\mu_{eo}$ is the electrosmotic mobility, $\zeta$ is the zeta potential, $e$ is the relative permittivity constant, and $\eta$ is viscosity. A reduction of the zeta potential has been observed when increasing ACN. This is due to ACN absorption to the capillary wall, which changes the electrical properties of the Stern layer and reduces the capillary surface charge [37]. ACN has a low permittivity value of 35.94 [38] that lowered EOF. Also the lower permittivity constant of ACN may have caused changes in the dissociation equilibrium of the dissolved compounds, thus modifying their electrophoretic properties. In contrast, ACN has a low viscosity of 0.341 MPa s [38] accounting for a theoretical increase of EOF. However, the effects of permittivity and zeta potential changes are greater as indicated by the decrease in EOF shown in Fig. 3. ACN also allowed a better separation between compounds and detection of a higher number of baseline-resolved peaks, likely due to the increase in solubility of the organic analytes. The most efficient ACN concentration was 15%, as higher concentrations did not improve separation but increased total analysis time and baseline instability.

To further improve separation, MEKC was also evaluated by adding SDS from 5 to 120 mM (data not shown). SDS micelles did not improve separation at concentrations below 20 mM. Above 20 mM resolution, baseline stability and repeatability were compromised. The same effect was
also reported in MEEKC studies [39]. Because the addition of SDS did not enhance separation of our sample’s compounds, addition of 1-butanol as a second organic modifier was evaluated due to its lower polarity that may dissolve low polar compounds. Increasing 1-butanol increased analyte migration time but improved resolution and increased the number of detected peaks by four compared with BGE with no 1-butanol as shown in Fig. 4. Decreased mobility was probably due to a decrease in EOF related to the higher viscosity of 1-butanol [38]. Increase in the number of peaks may be due to additional partitioning of the analytes in the different zones of the BGE. 1-Butanol has also been used in NACE and MEEKC modes [39]. However, its use in CZE is not very common. Up to 15% 1-butanol was tested but 9% was chosen because higher concentrations increased peak broadening probably due to longitudinal diffusion at low EOF [32].

### 3.3 NACE

NACE was also tested because the nature of our analytes of interest was unknown. In addition, NACE is particularly suitable for analytes that are not readily soluble in aqueous BGE [40]. Concentrations of ACN ranging from 5 to 50% in methanol were tested along with ethanol amide to increase the pH of the organic solution. Fifteen percent ACN and 20 mM ethanol amide provided the best results in NACE mode. Higher amounts of these compounds did not improve separation. EOF was reduced by increasing ionic strength with sodium acetate 20–50 mM to provide better separation. Sodium acetate resolved the peaks at 20 mM or higher as shown in Fig. 5. However, fewer peaks were detected by NACE in a 90-min run compared with CZE and none of the compounds detected in NACE were absent in CZE (compared by UV spectra of standard compounds run under both conditions). This suggests that the presence of water in the BGE improves the solubility of some important analytes. Also, water increases the permittivity constant of the BGE. Higher relative permittivity facilitates ionic dissociation and increases EOF [38]. In NACE systems ion–ion interactions are stronger and, in the cases where the relative permittivity of the BGE is below ten, ions tend to pair to neutral species making separation more difficult [38].

### 3.4 MEEKC

A common MEEKC BGE consists of a micro emulsion of hexane in a sodium borate aqueous solution aided by high concentrations of SDS and 1-butanol [39]. Optimum proportion of these reagents varies with the type of analyte. In this case, no separation was observed with SDS concentrations below 15%. Higher concentrations increased analysis time to over 90 min. Concentrations of 1-butanol below 20% yielded poorer emulsion stability and lower repeatability. Therefore, 15% SDS and 20% 1-butanol were chosen to form the
microemulsion with a 10 ppm sodium borate solution. Higher concentrations of sodium borate increased migration time and did not provide additional resolution. Hexane concentration was 0.8%, and as previously reported [39] its variation did not cause significant changes (data not shown). No significant differences ($p<0.05$) were found in chloroform extracts. Therefore, all further analyses were run using MWC extracts for which CZE gave the largest number of peaks and highest resolution (Fig. 6).

### 3.5 Selection of UV wavelength

For comparison purposes, 190 nm was chosen because most analytes of interest showed one maximum absorbance peak at this wavelength. Higher wavelengths are usually more specific [18] and, thus, very useful for targeted metabolomics analysis. However, assessing untargeted differences in biological systems requires the detection of as many metabolites as possible. For NACE experiments, the wavelength used was 205 nm because this is the UV cut-off of methanol in the BGE.

### 3.6 Compound identification and statistical analysis

A series of standards compounds were dissolved in the MCW mixture to simulate the sample preparation and run under the selected CZE conditions. A total of 36 HLB-infected and 18 healthy samples were run and peaks were automatically identified by the tool “library search” based on UV spectra and confirmed with migration time and UV spectra of pure standards run individually and by spiking the sample. Electropherograms were aligned before performing statistical analysis as recommended by García-Pérez et al. [27]. Alignment was performed using the “Align” tool in the 32 Karat software. Reference peaks for alignment were selected based on peak identity (migration time and UV spectra). We found this tool to be particularly suitable when a low number (e.g., 35 or lower) of peaks are detected with no major deviations in migration times. Normalization was done to the IS because other normalization methods usually give comparable results [27]. ANOVA of 36 PCR-confirmed-infected and 18 PCR-confirmed-healthy samples revealed six compounds present in significantly higher ($p<0.05$) concentrations in HLB-infected samples. Three of these compounds were identified by mobility and UV spectra as hesperidin, naringenin, and quercetin by our internal database and using pure standards. Electropherograms were aligned before performing statistical analysis as recommended by García-Pérez et al. [27]. Alignment was performed using the “Align” tool in the 32 Karat software. Reference peaks for alignment were selected based on peak identity (migration time and UV spectra). We found this tool to be particularly suitable when a low number (e.g., 35 or lower) of peaks are detected with no major deviations in migration times. Normalization was done to the IS because other normalization methods usually give comparable results [27]. ANOVA of 36 PCR-confirmed-infected and 18 PCR-confirmed-healthy samples revealed six compounds present in significantly higher ($p<0.05$) concentrations in HLB-infected samples. Three of these compounds were identified by mobility and UV spectra as hesperidin, naringenin, and quercetin by our internal database and using pure standards. However, unlike in orange juice, quercetin has not been previously detected in orange leaves by other methods. These six compounds were always in significantly higher ($p<0.05$) concentrations (154% or higher increase) in the infected samples as shown in Fig. 7. Similar results have been reported for other plants since Abu-Nada et al. [41] found a much higher proportion of compounds being up-regulated than those down-regulated in potato infected with Phytophthora infestans. In this study, we provide evidence that HLB induces production of hesperidin, quercetin, and naringenin. Hesperidin has been reported to increase in orange leaf during blight-induced zinc deficiency [42], suggesting its participation in the plant response-to-stress mechanism. Also, HLB may possibly be causing mineral deficiency through phloem obstruction in infected trees. However, this type of HLB secondary effects need to be further researched. Naringenin [43] and quercetin [44] have been reported to have microbial inhibition properties. Therefore, the plant probably synthesizes these compounds as a defense mechanism against pathogens. Genticic acid (free form) concentration was not significantly ($p>0.05$) different as shown in Table 1, suggesting that it may not be an effective biomarker. However, the glycoside form of genticic acid previously reported to increase in HLB-infected trees [13] was not detected in this analysis probably because...
of its low UV–Vis absorbance. Table 1 summarizes the migration time, mobility, wavelengths of maximum absorbance, and the potential identity of each detected metabolite. While spectra allowed tentative identification of hesperidin, naringenin, and quercetin, three other potential biomarkers remain unknown. Other analytical tools are required for their positive identification.

4 Concluding remarks

CE coupled with DAD analysis and CZE was able to detect significant differences in metabolite profile between healthy and HLB-infected citrus leaves. Results show CE-DAD potential for untargeted metabolomic analysis for plant diseases as well as its use for monitoring HLB infection based on the concentration of hesperidin, naringenin, and quercetin in MWC leaf extracts. A limitation of DAD is the difficulty in identifying compounds using their UV–Vis spectrum. Identification of unknown compounds from this research requires the use of alternative methods. However, biomarker discovery does not necessarily imply compounds identification. Similar changes in metabolite profiles produced by other stresses such as mineral deficiencies may be caused by factors other than HLB. Therefore, the potential biomarkers identified in this study may not be specific individually. However, their combined change in concentration relative to healthy levels and to each other may provide the needed specificity. Further research to demonstrate that the same levels of all identified compounds are not found in the tree leaves under other stress situations to determine biomarker specificity is needed and is underway in our laboratory. Also, a metabolomics time-controlled experiment is underway in a greenhouse with HLB-infected branches grafted into healthy young trees. This experiment will allow determining the earliest time necessary to detect differences in metabolite profiles and other changes at each stage of the disease.

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The authors have declared no conflict of interest.

5 References


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