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Hydroxychavicol and Eugenol Profiling of Betel Leaves from *Piper betle* L. Obtained by Liquid-Liquid Extraction and Supercritical Fluid Extraction

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ABSTRACT

The chemical components of liquid-liquid extraction (LLE) and supercritical fluid extraction (SFE) obtained from dried betel leaves (*Piper betle* L.) were determined and evaluated for the percentage extraction of hydroxychavicol (HC) and eugenol (EU). SFE was conducted at 60 °C and a working pressure of 8 MPa (SFE1) and 6 MPa (SFE2. The chemical profiles were determined using high performance liquid chromatography (HPLC) with a method developed and validated for quantitative determination of HC and EU in the extracts from betel leaves. The analysis was carried out using HPLC on a Hypersil GOLD column (C_{18} with 250 × 4.6 mm) using methanol-water (70:30 volume per volume) as the mobile phase under an isocratic system. The flow rate was 0.7 $mL.min^{-1}$ and the detection was evaluated at the wavelength of 280 nm with a UV detector. The validation using hydroxychavicol (HC) and eugenol (EU) as a standard demonstrated a linear relationship (correlation coefficient = 0.997 and 0.998, respectively), with precision (relative standard deviation < 4%) in the concentration range of 40–100 $\mu g.mL^{-1}$ For HC and EU, the limit of detection was 1.0 and 0.1$\mu g.mL^{-1}$, respectively, and the limit of quantification was 2.0 and 0.2 $\mu g.mL^{-1}$, respectively. The highest extraction yield (15.6% weight per weight, w/w) and a high content of HC (9.1% w/w) were obtained by using ethyl acetate refluxed extraction and LLE. A similar chemical profile, regarding the HPLC fingerprint showed quality consistency by SFE1 with lower extraction yields.

Key words: supercritical fluid extraction, *Piper betel* L., high performance liquid chromatography, hydroxychavicol, validation

INTRODUCTION

Betel vine (*Piper betle* L.) is a dioecious, evergreen creeper that grows in moist, tropical and subtropical regions where it is used widely as a traditional medicine in different countries such as Malaysia, Indonesia, Philippines, Thailand, China and many other western countries (Ramji *et al*., 2002). Betel leaf is an aromatic leaf belonging to the Piperaceae family commonly used as a masticatory as it is rich nutritionally and is known medicinally as a stimulant and carminative, an antiseptic and an expectorant (Philip, 1984). Its chlorophyll is beneficial in maintaining healthy teeth, clearing the mouth and throat and helping in digestion by encouraging salivation and

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neutralizing excess acid (Loranty *et al*., 2010). According to Duke (1985), the flavor of the leaf is due to the presence of essential oils (0.7–2.6%) and the other constituents are moisture (80–85.4%), carbohydrate (6.1%), protein (3%), fiber (2.3%), minerals and vitamins (2.3%), sugar (2.4–5.6%) and fat (0.8%). Betel leaves are perishable, yet they have a good market value both within and outside Asia. Due to limited storage time before quality is affected and the use of a poor transportation system, many betel leaves are disposed of as waste every year, while during the rainy season, leaf production is so high that the leaves remain unsold or sold at a throwaway price (Pin *et al*., 2009). Therefore, manufacturing of essential oil, talc, medicinal compounds, perfumes, beverages and food additives may be practical in stabilizing the market price of the crop year round. This requires the attention of researchers for the development of value-added products (Rayagura *et al*., 2007).

To date, the numerous studies carried out on the essential oil composition of *Piper betle* L. have identified five chemical groups depending on the predominance of particular compounds: (a) alkaloid/amide group (Stöhr *et al*., 2001); (b) propenylphenol group (Sarkar *et al*., 2008); (c) terpene/sesquiterpene group (Parma *et al*., 1997): (d) steroid group (Parmar *et al*., 1997); (e) prenylated hydroxybenzoic acid group (Flores *et al*., 2009). Hydroxychavicol (HC) and eugenol (EU) have been regarded as the major compounds belonging to the propenylphenol group. The chemical structures of HC and EU are shown in Figure 1.

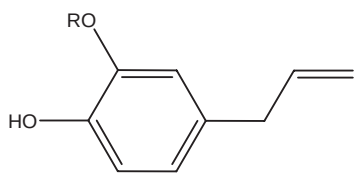

Figure 1 Chemical structure of hydroxychavicol (HC, R =H) and eugenol (EU, R= CH_3).

Extraction and product recovery were considered as the most essential steps in the evaluation of target molecules from various plant parts (Rayagura *et al*., 2011). Most of the extraction processes are time consuming, laborious and involve lengthy operational techniques, bulk amounts of solvents and ultimately result in thermal decomposition of the target molecules at continuously high temperature. The high temperatures used in the extraction method often lead to the degradation of heat-sensitive compounds. Moreover, traces of toxic solvents are rarely removed from the extracts, which directly influence the quality of the products. Therefore, alternative extraction techniques with better selectivity and efficiency are highly desirable. Products obtained by supercritical fluid extraction (SFE) are free from toxic residues and generally possess higher quality than products obtained by conventional techniques (Handley, 1999). Therefore, to fulfill the current pursuit of clean technology, it is necessary to demonstrate to investors that in addition to being technically viable, SFE is indeed an attractive choice for an extraction process. The composition of the extract in terms of its major compounds and one functional property would help the decision makers. The biological activity of extracts used as nutraceuticals must also be monitored (Singtongratana, 2012). To obtain the required information for process design, the identification of the solute mixture is necessary; therefore, the chemical composition of SFE extracts must be determined by appropriate methods, such as gas chromatography with a flame ionization detector, gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) or ultraviolet spectrophotometry.

The major compound contents from fresh or dried betel leaves by liquid-liquid extraction (LLE) of HC have been reported by either GC-MS or HPLC. Hydro-distillation (steam distillation) is generally used for obtaining volatile oils from plants. However, the high processing temperature

required can also lead to the degradation of heat-sensitive compounds, which may affect the 5-(2-propenyl)-1,3-benzodioxole content of 18.27% w/w based on fresh betel leaves, determined by GC-MS (Sugumaran *et al*., 2011). Moderate yields of HC extraction, based on fresh leaves, extracted in boiling water, reported 0.096% w/w determined by GC-MS (Tawatsin *et al*., 2006) and 5% w/w determined by HPLC (Pandey and Bani, 2010). The HC contents of dried-leaf ethanolic extraction were reported to be 0.9 % w/w (Sarkar *et al*., 2008) and 54.4% w/w (Bandyopadhay *et al*., 2011) determined by HPLC. Most reports were focused on the bioactivity of betel leaves but the effect of the extraction method on the dried leaves has yet to be studied. The present work focused on the effects of extraction techniques on the quality of betel leaf extracts. The contents of active compounds including HC and EU in the extracts of dried leaves were determined using HPLC. The chemical compositions of HC and EU subjected to two different extraction techniques were obtained from the HPLC fingerprint (Singkhonrat, 2006). The objectives of the present study were: (1) to develop methods that would speed up the extraction of *Piper* materials, (2) to optimize the recovery of hydroxychavicol (HC) and eugenol (EU) and (3) to provide repeatable and quantitative analysis and identification of the principal active components.

MATERIALS AND METHODS

Betel leaves were collected from the east of Thailand in Chachoengsao province and the neighboring provinces of Prachin Buri, Sa Kaeo and Chanthaburi. Fresh, healthy, green and matured betel leaves were collected in bulk and washed thoroughly in clean water to remove any dirt and dust present. The clean leaves were dried at 35−40 °C for 12 hr, ground to a powder using a grinder (mill machine, Pulverisett 14, Ider-Oberstein, Germany) and stored at 0−5 °C prior to use.

Liquid-liquid extraction and supercritical fluid extraction

The LLE extraction was carried out according to Singtongratana *et al*. (2012). Betel powder (50 g) was covered with ethyl acetate (125 mL) and refluxed for 20 min. The ethyl acetate slurry was shaken for 24 hr followed by suction through a Buchner funnel with Whatman No. 1 filter paper to remove any insoluble material. The filter cake was rinsed four times with 30 mL of ethyl acetate. The filtrate was transferred to a separatory funnel and washed twice with 75 mL of distilled water. The ethyl acetate fraction was separated and dried with anhydrous $MgSO_4$ and was refiltered as above. The filtrate was evaporated to dryness and the extract weighed (7.81 g, 15.6% yield).

Ten kg of betel powder was subjected to SFE using an SFE apparatus (24L-SFE, Guangzhou masson New Separation Technology Co., LTD China). Briefly, CO_2 (99.5% w/w pure) was delivered from a standard cylinder and compressed to an extraction pressure of 6–8 MPa by an air-driven liquid pump after cooling at a flow rate of 0.3 $L.hr^{-1}$. The CO_2, together with the betel powder, entered the extraction cell through a heat exchanger, where the system reached an extraction temperature of 60 °C at 8 MPa for 3 hr before ethanol was used as a elute and cosolvent for another hour to obtain products called SFE1. The SFE2 products were obtained in a different separator under the same temperature with reduced pressure of 6 MPa for 3 hr.

High Performance Liquid Chromatography analysis and conditions

The HPLC analysis (Singtongratana *et al*., 2012) of the extract samples was performed using an isocratic system equipped with an 1100 series HPLC pump (20AT; Shimadzu Corp.; Tokyo, Japan), manual sampler equipped with a 20 µL sample loop. The output signal was detected by a UV detector and integrated using the Microsoft Excel software package (Excel

97–2003; Microsoft, Redmond, WA, USA). The analysis was carried out using HPLC on a Hypersil GOLD column (C_{18} with 250 × 4.6 mm).

Method development

Optimization of parameters in the HPLC was carried out through investigating, the influence of the mobile phase and the flow rate. Various mobile phases (HPLC-grade methanol, acetonitrile and double distilled water) were tried in different ratios for selection. For the present work, a 70:30 v/v mixture of methanol and water was chosen as the mobile phase. The elution was performed under isocratic conditions at a flow rate of 0.7 $mL.min^{-1}$. Chromatograms were recorded at 280 nm.

Standard stock solution

Stock solutions of HC and EU were prepared by weighing each compound in a 100 mL volumetric flask containing 75 mL of methanol and then made up to the mark with methanol to get a concentration of 1000 $\mu g.mL^{-1}$. These stock solutions were further diluted to obtain the desired concentration.

Preparation of calibration curve

The above stock solution was diluted to obtain a concentration of 30, 40, 50, 60 and 100 $\mu g.mL^{-1}$. These different concentrations were injected into HPLC equipment. A calibration curve was prepared by plotting concentrations on the x-axis and the peak area on the y-axis (Table 1 and Figure 2).

Sample stock solution

A quantity of extract sample equivalent to 10 mg was taken in a 10 mL volumetric flask and the mobile phase was added up to the mark and filtered to get a concentration of 1000 $\mu g.mL^{-1}$. The solution was sonicated for 10 min and filtered. This solution was further diluted to obtain a concentration of 100 $\mu g.mL^{-1}$.

Procedure

Into a series of 5 mL volumetric flasks, 0.15–0.50 mL of the above stock standard solution (both HC and EU) were transferred. The total volume in each flask was made up to 5 mL with methanol and filtered through a 0.45 μm membrane filter. Initially, the mobile phase was pumped for about 30 min to saturate the column thereby establishing the baseline. Then, 20 μL of standard solution or of one of the sample solutions was injected three times. A quantitative determination of the active ingredients was made by comparison of the peak area from the sample injection to the corresponding peak area from a standard injection. The amount of HC and EU present in a sample was calculated using the standard calibration curve. The retention time of HC and EU was found to be 6.623 and 8.607 min, respectively.

Method validation

The method was validated according to Andlauer *et al*. (1999) for different parameters, assessed by linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ).

Linearity of response

To demonstrate the linearity of response, a series of solutions ranging from 30 to 100 $\mu g.mL^{-1}$ was made and injected into the HPLC system following the described conditions. The graph of concentration versus peak area was plotted and it was found that the correlation coefficient and regression analysis were within the limits (Table 1, acceptance criteria being that the correlation coefficient (r^2) should be more than 0.98.).

Accuracy

To establish the accuracy of the test method, sample solutions of SFE2 in triplicate by spiking the test solutions with HC and EU at 100 and 150%, respectively, were prepared and injected into the HPLC system as per the test

procedure. The 'amount added', 'amount found' and average % recovery for HC and EU at the 100 and 150% spike levels, respectively, were calculated and the results are summarized in Table 2 (acceptance criteria being that the mean recovery should be within 100±4%).

Reproducibility

The reproducibility was estimated according to Andlauer *et al*. (1999) by making repetitive injections of a standard mixture solution (100 μg.mL^{-1} for each) under the optimum conditions (n = 10). The results are shown in Table 3 (acceptance criteria being that the percentage relative standard deviation (%RSD) of the determinations should not be more than 6 for repeatability and reproducibility).

Limit of detection and limit of quantification

The LOD and LOQ for HC and EU were predicted based on the parameters of signal-to-nose ratio (σ) and the slope of the calibration curve (S), calculated from the linearity equation of HC and EU. The predicted values in micrograms per milliliter can be obtained by using the formulae 3 (σ) / S for LOD and 10 (σ) /S for LOQ. The values of LOD and LOQ were calculated. The LOD of HC and EU was found to be 1 and 0.1 μg.mL^{-1}, respectively; The LOQ of HC and EU was found to be 2 and 0.2 μg.mL^{-1}, respectively.

RESULTS

Optimization of the chromatographic condition

Typical chromatograms of the standards are shown in Figure 2, with hydroxychavicol (HC) at a retention time of 6.61 ± 0.05 min and euginol (EU) at a retention time of 8.64 ± 0.05 min. The calibration curve of the HC and EU provided all linearity parameters as shown in Table 1.

The linear regression for the HC and EU determination as a standard demonstrated a good linear relationship with a correlation coefficient of 0.997 and 0.998, respectively.

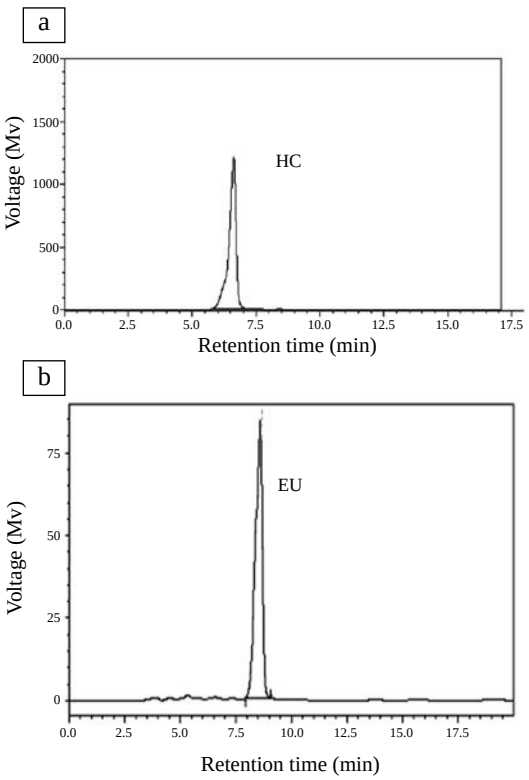

Figure 2 Chromatogram of (a) standard hydroxychavicol (HC) with concentration of 1,000 mg/L (shown at 6.623 min) and (b) standard euginol (EU) with concentration of 50 mg/L (shown at 8.607 min).

Table 1 Linearity parameters for the calibration curve of the hydroxychavicol (HC) and euginol (EU).

Compound	Slope (a)	Intercept (b)	(r^2)
HC	24167	-18105	0.997
EU	38454	-14875	0.998

Working range = 30–100 μg.mL^{-1}, r^2 = Correlation coefficient.

It was found that the accuracy of this method was 97.32% and 96.85% for HC and EU, respectively (n = 3) with %RSD values less than 3% (Table 2), the precision in terms of repeatability and reproducibility defined as %RSD values of the peak area were 0.08 and 0.28, and 0.11 and 0.25%, respectively for HC and EU (Table 3).

The chromatographic profiles of the extracts showed well-resolved peaks when the chromatographic conditions described in Figure 3 were employed. Peak 1 with a retention time of 6.71±0.28 min was identified as HC. Peak 2 with a retention time of 8.52±0.25 min can be assigned to EU.

Extraction yield

The extraction yield (expressed as the weight of extract relative to the weight of the dried plant material) of the oil obtained from the betel powder by LLE was 15.6±0.02% w/w, while the extraction yield of the SFE obtained was relatively lower than LLE (Table 4). The extraction yields of 8.0±0.20% w/w were collected in the first separator (extracts-1st separator, SFE1), and more impurities, but lower yields (0.5±0.14% w/w) were collected in the second separator (extracts-2nd separator, SFE2).

Table 2 Results of the recovery test for hydroxychavicol (HC) and euginol (EU) from supercritical fluid extraction (SFE) extract of *Piper betle* L.

Compound	Recovery (% ± SD)	Specification
HC	97.32 ± 0.6	100%
EU	96.85 ± 0.02	150%

Spiked concentration = 50 $\mu g.mL^{-1}$ of standard mixed to the sample n = 3.

Table 3 Precision data for determination of hydroxychavicol (HC) and euginol (EU) by high performance liquid chromatography analysis.

Compound	Repeatability (%RSD)[a]	Reproducibility (%RSD)[b]
HC	0.08	0.28
EU	0.11	0.25

[a] = n = 10 for each sample on same day.

[b] = 10 times for each freshly prepared sample n = 3 on same day.

Table 4 Comparison of extraction yields of hydroxychavicol (HC) and euginol (EU) by different methods.

Method	Condition	Yield (HC, EU % w/w)
Drying	37-50 ºC 12 h, RT 24 h	10%[a]
LLE (ERE)	Ethyl acetate refluxed 20 min + RT 24 h	15.6% (58, 6.28)[b]
SFE1	60 ºC, 8 MPa; CO_2 3 h CO_2 + Ethanol 1 h	8% (48.6, 5.66)[b]
SFE2	After SFE1 extraction, 60 ºC, 6 MPa; CO_2 + Ethanol 3 h	0.5% (24.2, 14.71)[b]

[a] = The yield is based on fresh leaves of *Piper betle* L.

[b] = The extracted yield is based on dried betel powder of *Piper betle* L. and the percentage content of HC and EU

RT = Room temperature, LLE = Liquid-liquid extraction, ERE = Ethyl acetate refluxed extraction, SFE = Supercritical fluid extraction.

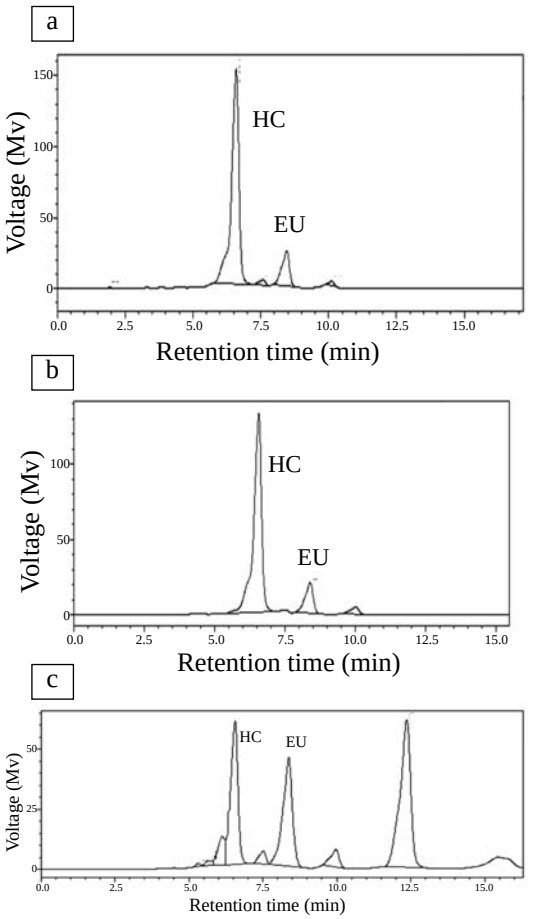

Figure 3 Chromatogram of hydroxychavicol (HC) and euginol (EU) in extracted oils of different extraction methods; (a) LLE (ethyl acetate refluxed extraction) with concentration of 112 mg.L^{-1}, (HC shown at 6.594 min and EU shown at 8.454 min) (b) supercritical fluid extraction first separator (SFE1) with concentration of 114 mg.L^{-1}(HC shown at 6.573 min and EU shown at 8.400 min) , (c) supercritical fluid extraction second separator (SFE2) with concentration of 130 mg.L^{-1}(HC shown at 6.560 min and EU shown at 8.373 min).

Comparison of liquid-liquid extraction and supercritical fluid extraction techniques on percentage extraction of hydroxychavicol and eugenol

A comparison of extraction under 60 °C (SFE, third entry in Table 4) and 77 °C (LLE, second entry in Table 4) showed that the higher temperature had the higher extraction yield. This could be explained as the effect of increased diffusion and fluidity of extracts from the matrix to the solvent. However, it should also be noted that SFE required a lower temperature than typical LLE methods. This characteristic is good for heat-sensitive materials, but the heat seems to have no substantial effect on HC and EU, in this case higher yields were obtained of both HC and EU for LLE (58 and 6.28% w/w, respectively). A comparison of the extraction yield under different pressures in SFE found that the higher pressure has the higher extraction yield. It is a reasonable observation that the density of the SFE is dependent on pressure which accelerated the solubility of the analyst (HC and EU) in carbon dioxide (CO_2) and ethanol. HC and EU have a good solubility in a supercritical fluid (SF) such as CO_2 similar to ethyl acetate as shown in the chromatogram of SFE1 in Figure 3b. However, when ethanol was used as a co-solvent for a longer period (3 hr) in the extraction process, SF of these co-solvents at 6 MPa also extracted higher polar components as minor impurities, shown at retention times of 5.322 5.617 and 6.121 min, along with lower polar components as major impurities, shown at retention times of 9.966 and 12.359 min in the chromatogram of SFE2 in Figure 3c.

DISCUSSION

The effects of extraction methods on the quality of betel leaves (*Piper betle* L.) was investigated based on the content of two compounds—hydroxychavicol and eugenol (Pin *et al*., 2011). The highest extraction yield

(15.6% w/w based on betel powder) and a high content of HC and EU (58% w/w and 6.28% w/w, respectively; calculated for 9.1% w/w and 0.98% w/w, respectively, based on betel powder) were obtained by using ethyl acetate refluxed extraction, LLE. SFE1 can be an alternative for obtaining similar quality extracts from betel leaves (*Piper betle* L.) with half the amount of yield extraction and a similar chemical profile compared with LLE. Moderate extraction yields (8% w/w based on dried betel leaves) and a high content of HC and EU (48.6% w/w and 5.66% w/w, respectively) were obtained by SFE1. Chromatographic analysis indicated that the chemical compositions of the LLE, SFE1 and SFE2 extracts differed qualitatively and quantitatively (Hopfgartner *et al*., 1990). The SFE2 chromatogram showed some amounts of other components that were not quantified due to the lack of standards, which indicated higher level of impurities with the lowest yield in this case. Overall, the chemical profiles of SFE were influenced by the extraction pressure and co-solvent used in this research. Furthermore, with the extracts obtained in the second separator, the pressure did affect the lower extraction yield and quality of extracts with the number of impurity peaks in the chromatogram.

Interestingly, the extract obtained by SFE1 showed a similar chemical profiling and evaluated quality as LLE according to the chromatogram fingerprint in Figure 3. However, the influences of process parameters on the extraction yield are under investigation along with the particle size of the betel material. In addition, the application of different solvents on the dried-leaf powder might lead to the successful extraction of valuable phytochemicals in this herb. This work has shown that supercritical fluid extraction (SFE1) had no influence on the quality of dried betel-leaf extracts, but obtaining better yields must be studied further. Therefore, a great concern regarding the quality of this extract has been overcome by using supercritical fluid extraction as a greener and cleaner technology. However, its high cost is still a vital issue that must be addressed before SFE can be accepted in commercial production.

CONCLUSION

Supercritical fluid extraction was developed as a method to speed up the extraction of *Piper* materials with optimization of the recovery of hydroxychavicol (HC) and eugenol (EU) to ensure the quality of the extract regarding its HPLC fingerprint. The proposed HPLC method was found to be simple, precise, accurate and rapid for the determination of HC and EU in the extracted sample. The sample recovery from all extracts was in good agreement with the limits. Hence, this method can be easily and conveniently adopted for routine analysis of HC and EU in quality control.

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