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## **Postharvest caffeoylquinic acids accumulation in forced chicory roots: insights into the role of temperature, water loss, and biological defence mechanisms**

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## **Abstract**

Forced chicory roots (FCR), a by-product of Belgian endive cultivation, are a valuable source of bioactive phenolic compounds such as caffeoylquinic acids (CQAs). This study investigated how post-harvest treatments, including wounding and drying, could be used to increase CQAs content in FCR. Results showed that wounding and water loss treatments significantly increased the content of CQAs in FCR, with chlorogenic acid (5-CQA) content being 3.3-fold higher after wounding and 244 h of drying at room temperature, and dicaffeoylquinic acids (diCQAs) content being 2.3-fold higher after wounding and 71 h of drying at room temperature. The mechanism behind the accumulation of CQAs in FCR was also investigated, with two potential competitive mechanisms proposed: biosynthesis of CQAs by the phenylpropanoid pathway (PP) and consumption/degradation by oxidation/oligomerization reactions (OPO). These mechanisms are temperature- and water-content dependent.

Keywords: chlorogenic acid; dicaffeoylquinic acids; wounding; biosynthesis; optimization; forced chicory roots

## 1. Introduction

Plants offer, through their metabolism, many beneficial compounds for human health. Thus, natural plant extracts containing these compounds are increasingly used in the food, pharmaceutical and cosmetic industries. However, the production of these extracts by conventional methods such as solid-liquid extraction has low profitability and a strong ecological impact due to the limited content of these compounds in plants. An alternative to overcome these disadvantages is to increase the content of these metabolites during the growth phase or the post-harvest phase. The post-harvest application of abiotic stress (wounding, UV, drought or chemical elicitation) is well-known to increase the content of phenolic compounds of some vegetables such as potatoes, carrots or globe artichokes <sup>1-6</sup>.

However, abiotic stress on Belgian endive (*Cichorium intybus* var. *foliosum*) or its by-products have never been investigated so far. Belgian endive is a typical food of Belgium and France, mainly eaten as salads or gratins, and its production is located in the north of France, in Belgium and in the Netherlands. In France, more than 140,000 tons are produced each year<sup>7</sup>. During the cultivation process, many residues are produced, forced chicory roots (FCR) being the main one. Usually, FCR are used for feed <sup>8</sup> or composting as biowaste. However, FCR are a great source of phenolic compounds and particularly chlorogenic acid (5-CQA) and dicaffeoylquinic acids (diCQAs) <sup>9,10</sup>. These caffeoylquinic acids (CQAs) have valuable bioactivities such as antioxidant, antibacterial activity and choleric properties<sup>11-14</sup>. Moreover, FCR is a raw material that did not undergo any thermal or chemical treatment during the cultivation process. Thus, the biological system of the biomass is still active and reactive to stimuli. Pre and post-harvest abiotic stress can be highly interesting as it will activate the plant's defence mechanisms. The main reaction of the plant is the activation of its secondary metabolism, which leads, among other things, to the production of phenolic compounds <sup>15,16</sup>. Briefly, reactive oxygen species (ROS) content leads to the activation of the phenylpropanoid (PP) secondary

metabolism<sup>17</sup>. Increasing the ROS content enhances phenylalanine ammonia lyase (PAL) activity, a key enzyme that controls the transformation of phenylalanine into cinnamic acid<sup>18,19</sup>. PAL enzyme is the key control point for 5-CQA formation in the PP pathway<sup>20</sup>. Activation of PAL by ROS increasing is called secondary response<sup>21,22</sup>. In this article, two abiotic stresses are investigated for the co-benefits they bring: drying and wounding by cutting after harvest.

Drying fresh biomass is useful to stabilize it, to avoid enzymatic degradation of polyphenols<sup>23</sup> and this can also influence the phenolics content<sup>24–26</sup>. It has contradictory effects depending on biomass, drying temperature or water loss speed<sup>25,27–29</sup>. For example, phenolic compounds can be involved in biological pathways which produce phenolic compounds (e.g., the PP pathway) or pathways which consume those compounds such as lignin synthesis, Maillard reaction or reaction with amino acids. Thus, it is difficult to know which pathway is predominant or important<sup>30,31</sup>. To accelerate the drying process, the biomass can be cut into pieces. However, this will wound the biomass and thus activates its defence mechanisms. Doing so, the phenolics content can also vary after a few minutes to a few hours<sup>32</sup>. The objective of this paper is to determine how wounding and drying conditions impact the accumulation of 5-CQA and diCQAs in the FCR. The article provides insight into how and to what extent the application of abiotic stress could enhance the CQAs content in the FCR.

## **2. Material and methods**

### **2.1. Raw materials**

The experimental station of APEF (l'Association des Producteurs d'Endive de France, Arras, France) supplied Forced Witloof Chicory Roots (*Cichorium intybus L.*) of the cultivars "Flexine – Vilmorin" and "Laurine". For each protocol, around 1 kg of FCR was used. These FCRs were subjected to a thorough washing with cold tap water and subsequently peeled.

### **Analytical reagents and chemicals**

Thermo Fisher (Illkirch, France) was the source of the following chemicals: ethanol (99.9%), acetonitrile (99.9%), formic acid (98-100%), and ascorbic acid (>99%). Methanol (99%), Diméthylsulfoxyde (DMSO) (99.9%) were provided by VWR (Fontenay-sous-Bois, France). Diphenyleneiodonium chloride (DPI) (>98%) was purchased from Sigma-Aldrich (Saint-Louis, USA). Ultra-pure water come from a Milli-Q system (Millipore Corporation, USA). Chlorogenic acid standard (5-CQA) (>98%) was sold by Sigma (Saint-Quentin-Fallavier, France) and 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) (>98%) was obtained from Carbosynth (Compton, United Kingdom).

## **2.2. Experimental strategy**

### **2.3.1 Wounding stress application**

Two different wounds have been performed, shredded in julienne (2\*3\*40mm) with a manual shredder or cut in slices (2 mm thickness) with a manual slicer. With these wounds, peeled and shredded roots (R1) or peeled and sliced roots (R2) were obtained. After cutting, FCR were mixed and randomly separated on each experimental condition.

### **2.3.2 Drying and storage treatment**

R1 were dried in an oven UN160 with natural convection (Memmert, Rosny-sous-Bois, France) at three different temperatures (30, 40, 50 °C) until water content (WC) was below 10%.

Two drying kinetics were performed: at 30 °C with R1 and at room temperature with R2 to determine the effect of water loss. A storage kinetic was also performed at 4 °C with R2. Extractions and analyses were performed regularly to determine CQAs concentration all along the experiment.

### **2.3.3 Enzymatic inhibition by diphenyleneiodonium chloride (DPI) application**

DPI application procedure was derived from <sup>33</sup> protocol. Briefly, R1 were dipped during 2 min in a solution containing a DPI solution at 315 µM under agitation. The DPI solution was

prepared with 1% DMSO in ultra-pure water. Control roots were dipped during 2 min in a solution made with a 1% DMSO in water alone.

## **2.4 Analytical measurements**

### **2.4.1 Moisture determination**

As previously described<sup>34</sup>, the moisture content (MC) of FCR was determined in triplicate using a MB-35 moisture balance (Mettler Toledo, Viroflay, France). Approximately 1g of FCR was heated at 160 °C until a consistent weight was achieved. The calculation of WC (%) was performed according to the following formula:

$$WC = \frac{m_i - m_d}{m_i} * 100 \quad (\text{Eq. 1})$$

here,  $m_i$  represents the mass of the initial sample, while  $m_d$  denotes the mass of the dried sample. All following results are reported in dry weight to avoid any misinterpretation of data due to the concentration of compounds during drying.

### **2.4.2 Extraction procedure of CQAs**

Extractions were performed during 30 min in a 250 mL three-neck flask with septum to avoid solvent loss. The solvent was an ethanol-water mixture (70% v/v), the temperature was maintained at 70 °C and the solid: liquid ratio was fixed at 1/100 (g dry weight/ g solvent) in order to extract all CQAs as described in our previous paper<sup>35</sup>. In all experiment, the agitation speed was maintained at 400 rpm. Each extraction was made with 10 g of fresh biomass.

### **2.4.3 Quantification of the CQAs**

Quantification of CQAs was performed using our protocol previously described<sup>34</sup>. The quantification of the CQAs was carried out using Ultra-High-Performance Liquid Chromatography (UHPLC; Ultimate 3000; Dionex, ThermoFisher), which was equipped with a diode array detector DAD 3000. The measurement data was acquired and processed by the

Chromleon 6.8 software. The CQAs were separated using an Accucore AQ-C18 column, which was heated at 48°C. The mobile phase was composed of 1% formic acid in water (solvent A) and acetonitrile (solvent B), and the gradient was as follows: 0-1 min with 2% B, 1-5.5 min from 2 to 10% B, 5.5-7 min from 10 to 15% B, 7-8.5 min with 15% B, 8.5-11 min from 15 to 25% B, 11-13.5 min from 25 to 80% B, 13.5-14 mins from 80 to 2% B, and 14-15 mins from 80 to 2% B. The flow rate was maintained at 0.8 mL/min, and the sample injection volume was 1 µL. The UV acquisition was carried out at 320 nm. The identification and quantification of 5-CQA and diCQAs (as 3,5-diCQA equivalent) were achieved by comparing their relative retention times with the corresponding standard compounds. The diCQAs content were the addition of 3,4-di-caffeoylquinic acid, 4,5- di-caffeoylquinic acid and 3,5-diCQA. Due to initial variation of CQAs concentration in fresh biomass, all results are normalized relatively to initial concentration in mg/g dry matter (DM). Roughly, 5-CQA concentration in fresh FCR was between 0.5 to 1.5 mg/g<sub>DM</sub> and diCQAs concentration was between 1.5 to 2.5 mg/g<sub>DM</sub>.

#### **2.4.4 Extraction and quantification of total lignin**

The determination of total lignin content followed the Klason lignin procedure<sup>15,36</sup>, with some modifications. Shortly, 1 g dry weight of R1 were treated with 15 mL of sulphuric acid 95% at room temperature for 2 h and stirred regularly to ensure homogeneous mixture. The hydrolysate was put into a bottle and diluted with 460 mL of distilled water. The mixture was autoclaved at 120 °C for 1 h. The acid insoluble lignin was collected with a Whatmann (Maidstone, United Kingdom) glass microfiber GF/F in a filtration set up. Solid residue was then dried at 105 °C overnight and weighted. For acid soluble lignin, the filtrate was diluted by 10 with ultra-pure water and the absorption was measured at 240 nm with a UV-vis spectrometer (Agilent Technologies Cary 60, USA). An extinction coefficient of 25 L.g<sup>-1</sup>.cm<sup>-1</sup> for lignin was used to calculate the concentrations in accordance with<sup>37</sup>.

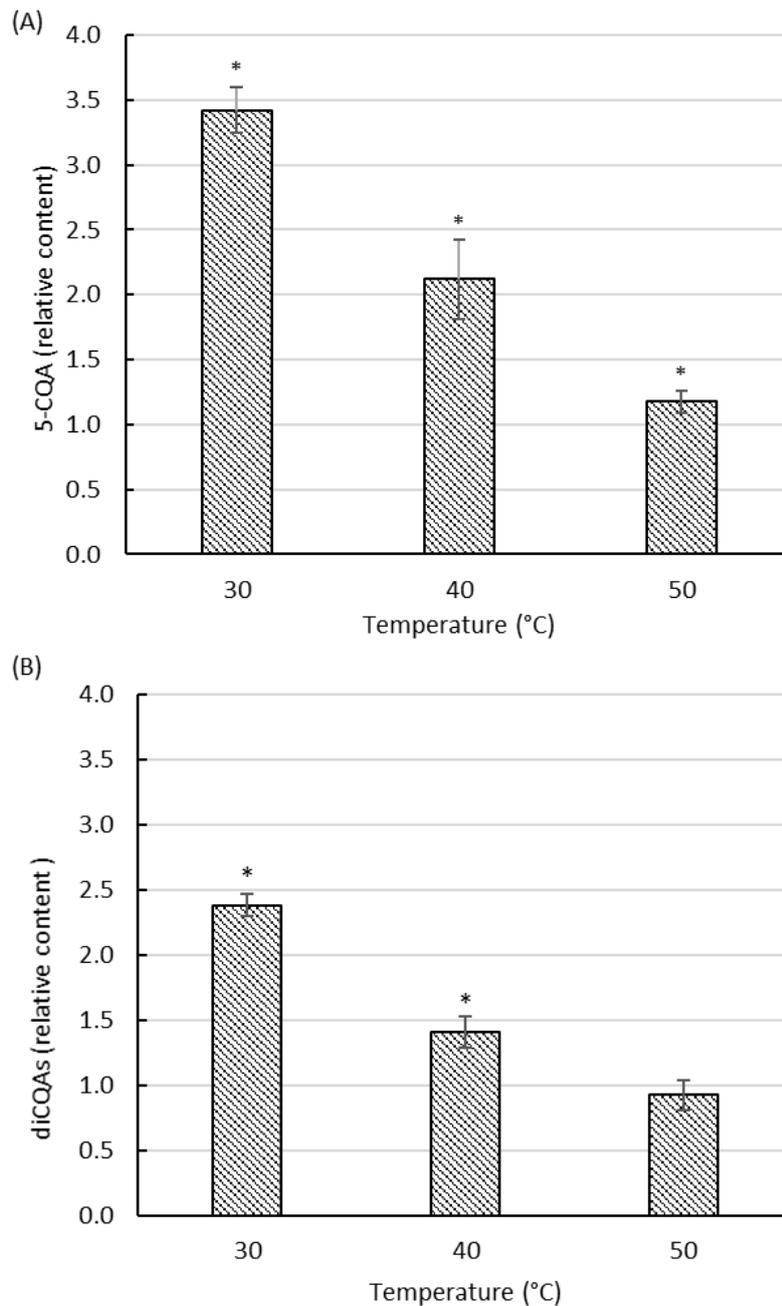
The concentration of lignin was measured in mg per g of dry matter (mg/g<sub>DM</sub>), and it accounted for both soluble and insoluble lignin, which were combined to obtain the total lignin content.

## **2.5 Statistical analyses**

All extractions were performed in triplicate. The average values and the standard deviation were calculated with Excel. Student tests were also performed with Excel to evaluate the significance of results ( $p = 0.05$ ).

## **3. Results and discussion**

**3.1 Effect of the drying temperature on CQAs accumulation of wounded FCR**  
FCR cut in julienne (R1) were dried at different temperatures (30, 40 and 50 °C) until the WC was under 10%. Drying took 36 h to achieve a WC under 10% for 40°C and 50°C but 96 h was required for the drying at 30°C. Extractions were performed on fresh and dried biomass. 5-CQA and diCQAs content for fresh and dried R1 are presented in Figures 1.



*Figure 1: Chlorogenic acid (5-CQA) (A) and dicaffeoylquinic acids (diCQAs) (B) relative content after drying at different temperature. All data are given as mean  $\pm$  standard deviation ( $n = 3$ ) with significant differences \* ( $p < 0.05$ ) between fresh and dry samples.*

Drying at 30, 40 and 50 °C has increased the amount of 5-CQA. The greatest increase (+ 242%) is noticed for a drying temperature of 30 °C. The content of diCQAs has increased by +138 % and +41 % after drying at 30 °C and 40 °C, respectively, but has remained constant at 50 °C. A high temperature can cause thermal degradation or isomerization of polyphenols<sup>28</sup>, thus this can occur here at 50 °C. The most interesting temperature to enhance the content of CQAs is 30 °C.

Indeed, at this temperature, 5-CQA content has increased 3.4-fold and diCQAs content has increased 2.4-fold. In order to understand how and when drying improves CQAs content, kinetic drying at 30 °C was performed (Figure 2).

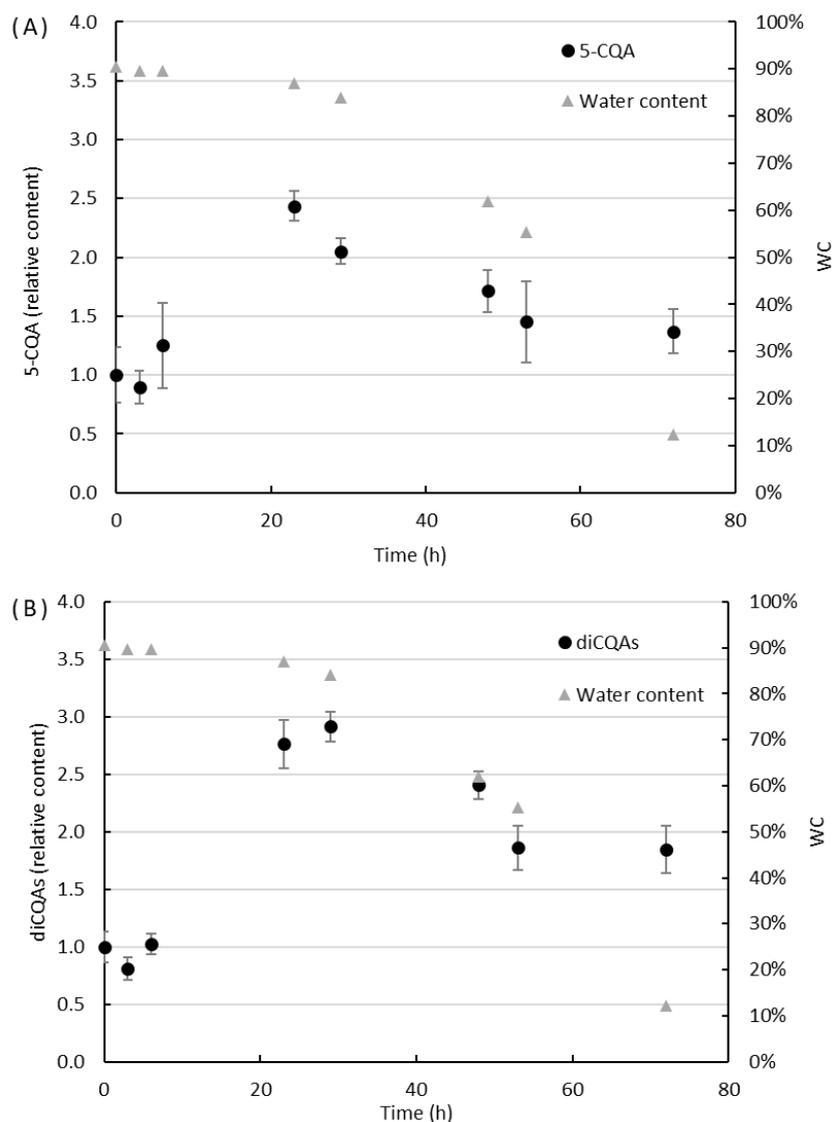


Figure 2 Chlorogenic acid (5-CQA) (A) and dicaffeoylquinic acids (diCQAs) (B) relative content during drying at 30 °C for R1. Results are given as mean  $\pm$  standard deviation ( $n = 3$ ).

The relative contents of CQAs were significantly increased ( $p < 0.05$ ) within the first 24 h. During this period, the loss of water is low (around 0.15% per hour) and the amounts of 5-CQA and diCQAs have been enhanced by 250% and 280%, respectively. This low water loss is caused by the oven used, with natural convection. Indeed, during the first 24 h, the oven

atmosphere was water saturated, so the FCR drying only began after humidity evacuation. From 29 to 53 h, the water loss became higher (1.19% per hour) and the contents of CQAs decreased significantly ( $p < 0.05$ ). At the end of the drying, when the WC is around 10%, the 5-CQA and diCQA content are 1.4-fold and 1.85-fold higher than those of fresh FCR.

In summary, two main interesting phases have been identified in the evolution of the content of CQAs: an increase during the first 24 h coupled with a low water loss and a decrease related to a high-water loss during the drying. The first phase can be explained by biological phenomena. This hypothesis will be investigated in 3.2. Indeed, some studies suggest that the drying of wounded biomass at room temperature leads to the biosynthesis of 5-CQA and diCQAs *via* the PP<sup>5,15,38,39</sup>. Thus, PP activation could be an explanation for this increase. During the second phase, the decrease in CQAs could be explained by another biological effect: lignification. Indeed, some authors have linked this decrease to a lignification process which converts CQAs into lignin and suberin<sup>15,40</sup>. For example, an increase of the relative expression of the related lignification enzyme (caffeoyl-CoA 3-*O*-methyltransferase, cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase) and a higher lignin content have been observed in carrots with water stress<sup>15</sup>.

### **3.2 Biological and oxidation explanations on CQAs evolution on FCR**

To evaluate whether the PP activation is the reason for CQAs increase, inhibition of ROS and its impact on CQAs content was evaluated during 96 h of storage at 30 °C. To do so, DPI, a known inhibitor of ROS, was used to measure the impact of ROS on the content of CQAs during drying. Results are shown in Figures 3.

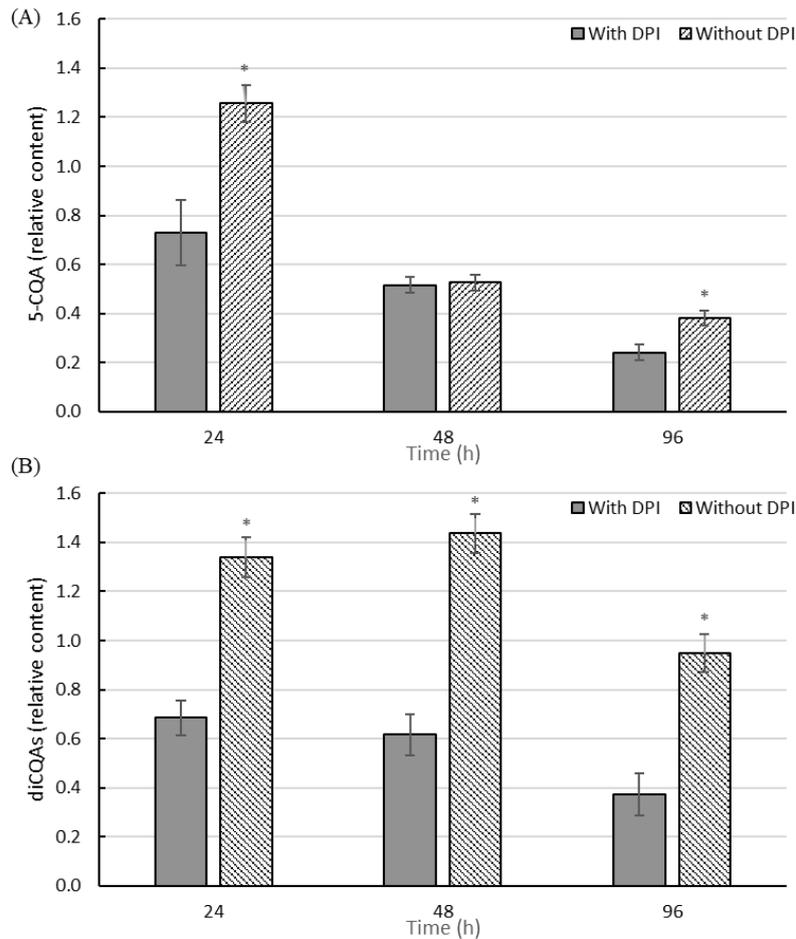


Figure 3: Relative content of chlorogenic acid (5-CQA) (A) and dicaffeoylquinic acids (diCQAs) (B) for R1 treated with or without DPI inhibitor. From T+24 to T+96h results are given as mean  $\pm$  standard deviation ( $n = 3$ ) with significant difference \* ( $p < 0.05$ ) between with or without DPI.

R1 treated with DPI showed a significant decrease in 5-CQA and diCQAs contents during the first 24 h, whereas R1 without inhibitor exhibited a significant increase in their contents. As DPI treatment inhibits the biosynthesis of CQAs, one can assume that the increase in CQAs content is due to the activation of the PP pathway. Without inhibitor after 48 h, 5-CQA content decreases as it is consumed to form diCQAs whose content remained unchanged. DPI inhibitor also seems to prevent this phenomenon as diCQAs content decrease. These findings have also been described in the literature for blueberries and carrots<sup>33,41</sup>. This pathway in the FCR seems to be similar to that observed in other vegetables and explains well the results observed herein.

As previously discussed, CQAs depletion could be due to the lignification process which consumes phenolic acids to produce lignin <sup>42</sup>. To verify this hypothesis, lignin content during drying at 30 °C with or without DPI inhibitor was measured. The results are shown in Figure 4.

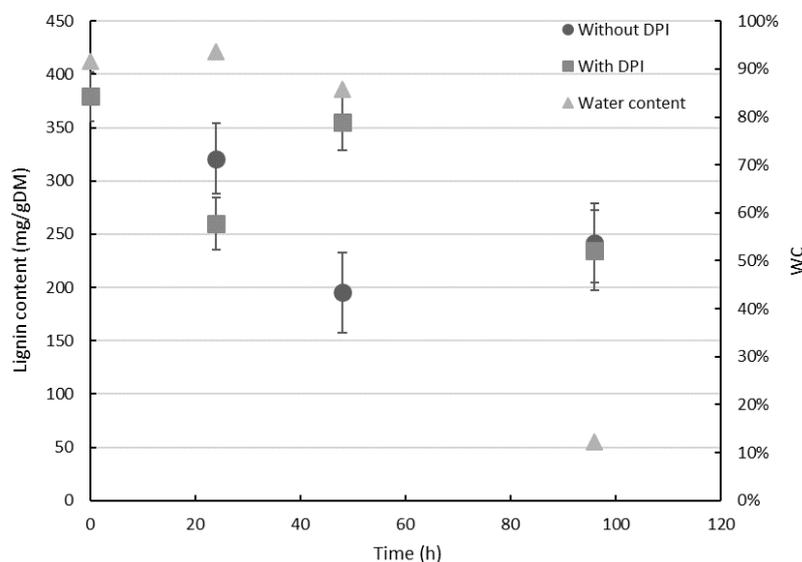


Figure 4: Total lignin content during drying at 30°C (in mg/g<sub>DM</sub>) for RI treated with or without DPI inhibitor. results are given as mean ± standard deviation (n = 3)

The lignin content was significantly affected by drying with or without DPI inhibitor ( $p < 0.05$ ) with a decrease of around 38% during the process. Moreover, no evident correlation can be made between lignin content and CQAs depletion seen before. Thus, the lignin hypothesis does not explain the decrease in the CQA content here for the FCR. It is worth mentioning that the conversion of CQAs (in particular 5-CQA) into lignin is still a controversial topic nowadays <sup>43</sup>. Other authors have linked CQAs depletion to oxidation and oligomerization <sup>44,45</sup>. Indeed, CQAs can be oxidized to CQAs-o-quinone by polyphenol oxidase (PPO) or by basic condition <sup>46</sup>, then the quinone reacts with other CQAs or proteins to produce condensation products (e.g., dimer, oligomers). pH of FCR is acidic (pH=6) <sup>47</sup>, so chemical oxidation caused by basic condition not seem relevant here and PPO activity is the privileged hypothesis. Oxidation and oligomerisation reactions lead to the formation of a yellow-brown compound called allomelanin <sup>48</sup>. This

hypothesis could be relevant for FCR as fresh biomass turns brown and pink a few hours after wounding and storage at room temperature (Figure 5A). This colour change could be due to CQAs/CQAs-o-quinone. Indeed, CQAs-o-quinone are reactive species and oligomerize to form brown compounds <sup>49</sup>. Additionally, caffeic acid quinone is known to be a pink coloured molecule <sup>50</sup>. Moreover, when the “brown-pink FCR” is placed during 5 min in presence of an ascorbic acid solution (1 g/L) a quinone reducing agent, the FCR bleaches to its initial colour (Figure 5B). Only the brownest part remains.



*Figure 5: (A) RI FCR after a few h at room temperature in a room (B) RI FCR few minutes after ascorbic acid treatment at room temperature*

FCR were dried with a paper towel and left at room temperature. The pink colour comes back within a few minutes. Taking into account that ascorbic acid is known to be a quinone reducer and that CQAs and caffeic acid are colourless compounds <sup>48</sup>, one can then assume that PPO was active on the FCR and that the conversion of CQAs into CQAs-quinone and their subsequent oligomerization could have occurred. Moreover, the FCR become browner during drying. Thus, CQAs content in FCR could be the result of two antagonist mechanisms: the biosynthesis of CQAs by the PP and the consumption of CQAs by oxidation/oligomerization (OPO). These two elements could explain the level of CQAs content in Figures 2. First, the

increase in CQAs during the first 24 h (PP biosynthesis > OPO), and after the decrease in CQAs (OPO > PP biosynthesis). The critical point seems to be when the WC begins to drop. Indeed, water activity is a critical point for the activity of enzymes involved in PP and OPO<sup>15,45</sup>. When biosynthesis is deactivated (Figure 5, with DPI inhibitor), only OPO occur and CQAs steadily decrease. Thus, to optimize the CQAs content, water losses should be managed.

### 3.3 Effect of the water loss on CQAs accumulation on wounded FCR

To avoid water loss, another type of cutting was used, sliced FCR (R2), and followed by a storing at 4 °C. The WC remained constant during the storage and the results are shown in Figures 6.

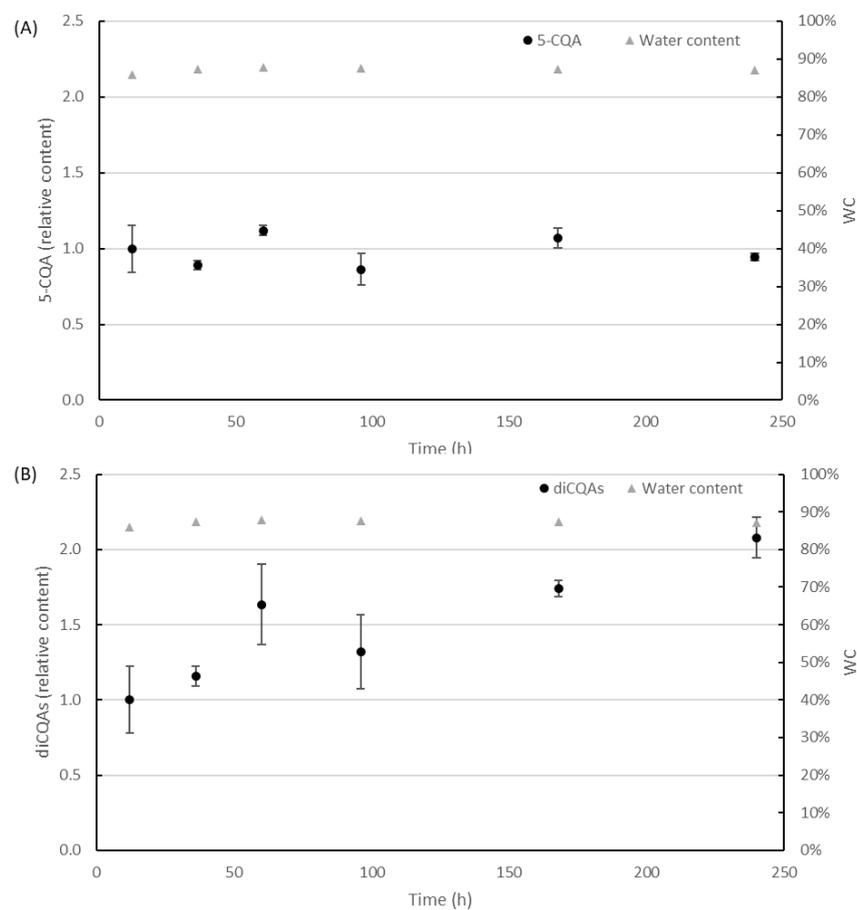


Figure 6: Chlorogenic acid (5-CQA) (A) and dicaffeoylquinic acids (diCQAs) (B) relative contents during storage at 4 °C for R2. results are given as mean  $\pm$  standard deviation (n = 3)

During the storage, the content of diCQAs showed a continuous increase ( $p < 0.05$ ) and a doubling after 250 h whereas 5-CQA contents are statistically equivalent ( $p > 0.05$ ). The R2 FCR was also dried at room temperature after wounding to evaluate the impact of water loss. The results are presented in Figure 7.

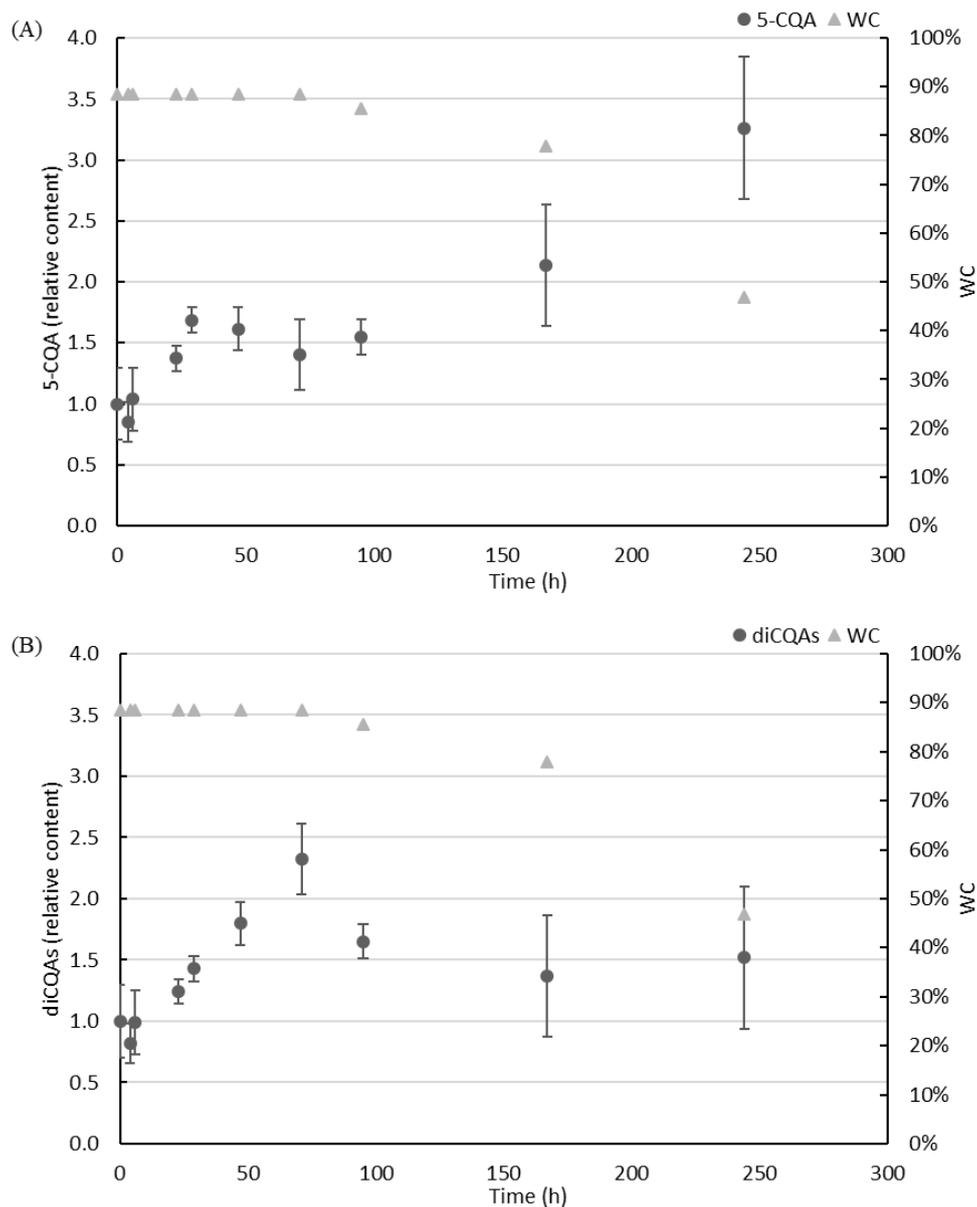


Figure 7: Chlorogenic acid (5-CQA) (A) and dicaffeoylquinic acids (diCQAs) (B) relative content during drying at room temperature for R2. results are given as mean  $\pm$  standard deviation ( $n = 3$ )

As observed for the previous experiment at 4 °C, without water loss, diCQAs content increases linearly ( $p < 0.05$ ) whereas 5-CQA content remains stable after 71 h ( $p > 0.05$ ). Thus, in the FCR,

wounding stress without any water loss leads to the biosynthesis of diCQAs rather than 5-CQA. These results are in accordance with the literature. Indeed, in the globe artichoke, rich in 5-CQA and diCQAs, as the FCR studied, abiotic stress leads to the accumulation of diCQAs instead of 5-CQA<sup>51</sup>. However, for potatoes, containing very low or undetectable diCQAs content, a wounding stress is known to produce only 5-CQA<sup>2,52,53</sup>. The hypothesis is that an enzyme that produces diCQAs is present in globe artichoke and FCR but not in potatoes. This enzyme could be the quinate hydroxycinnamoyl transferase (HQT) which produces diCQAs from 5-CQA<sup>43,54</sup>. Thus, under the effect of abiotic stress, 5-CQA could be converted into diCQAs by HQT, thus becoming an intermediate and not the final product if the biomass has a high content of HQT in the plant cells. After 71h at room temperature, WC of FCR began to fall. 5-CQA content is more than doubled during the drying process ( $p < 0.05$ ) whereas diCQAs content is decreased ( $p < 0.05$ ). At the same time, the WC decreased from 88.5% to 46.9% (-0.24% per hour). These results can be explained by the inactivation of the HQT in the vacuoles due to a water loss. Indeed, when plant cells are faced with water loss, the first reaction is to release water from vacuole to protect cytoplasm and cell walls<sup>55</sup>. The HQT reaction only occurs in vacuoles, so drying can cause vacuole dehydration and lead to the deactivation of HQT<sup>54</sup>. Under these conditions, 5-CQA, which is synthesized in the cytoplasm<sup>54</sup>, becomes the final product, as observed for carrots and potatoes<sup>5,56</sup>. Moreover, the water loss was slower than at 30°C (Figure 2), so the PP is still higher than OPO, which could explain the 5-CQA increasing during the drying. The increase of both 5-CQA and diCQAs in figure 2 could also be understood with a partial thermal inactivation of HQT at 30°C which leads to an increase of 5-CQA caused by a production not fully converted in diCQAs by HQT. To conclude, fine tuning of the WC and water loss rate of the biomass is a key factor to produce diCQAs and/or 5-CQA. In carrots, it was shown that the water stress induced more consumption than biosynthesis of polyphenols. At the opposite, wounding, leads to the increase of polyphenols content<sup>15</sup>. These conclusions

were in accordance with assumptions from FCR, an increase of CQAs when WC was controlled whereas a water stress lead to CQAs degradation. Nevertheless, with fine water control this CQAs-rich biomass would be a useful substrate to produce valuable antioxidants with potential applications in the pharmaceutical, cosmetic or food industries.

### **Abbreviations**

5-CQA, chlorogenic acid; CQAs, caffeoylquinic acids ; diCQAs, dicaffeoylquinic acids; DM, dry matter; DMSO, diméthylsulfoxyde; DPI, diphenyleneiodonium chloride; FCR, forced chicory roots; HQT, quinate hydroxycinnamoyl transferase; OPO, oxidation/oligomerization reactions; PAL, phenylalanine ammonia lyase; PP, phenylpropanoid pathway; R1, peeled and shredded roots; R2, peeled and sliced roots

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### **Conflict of interests**

The authors affirm that they do not possess any known financial interests or personal relationships that could have potentially influenced the findings presented in this paper.

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