Abstract: Background: *Styrax tonkinensis* is an economic tree species with high timber, medicine, oil, and ornamental value. Its seed, containing a particularly high oil content, are widely studied for their biodiesel properties by nutritional components and oil body ultrastructure. However, their comprehensive biochemical compositions have not been studied. Methods: During *S. tonkinensis* kernel development, we collected samples from four time points for metabolite profiling and classification through gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. Results: A total of 187 and 1556 metabolites were obtained, respectively. All of the metabolites were grouped into 19 and 21 classes by their chemical properties and into 8 clusters based on their change trends, respectively. Among all the metabolites, carboxylic acids and derivatives, flavonoids, fatty acyls, glycerophospholipids, organooxygen compounds, prenol lipids, and steroids and steroid derivatives were the main components. Alanine, glutamine, tryptophan, tyrosine and valine were the five most abundant amino acids. Palmitic acid, stearic acid, oleic acid and linoleic acid were the four major free fatty acids. Flavans, flavonoid glycosides and o-methylated flavonoids were the three major flavonoids. The differential metabolites distributions between different time points were identified. A pathway enrichment was performed, which was mainly focused on three groups, amino acids metabolism, carbon flow from sucrose to lipid and secondary metabolites biosynthesis. Conclusions: It’s the first time to analyze the metabolite fingerprinting for developing *S. tonkinensis* kernels and identify varied kinds of flavonoids. We performed metabolite profiling, classification and pathway enrichment to assess the comprehensive biochemical compositions. Our results described the change in major metabolites and main metabolic processes during *S. tonkinensis* kernel development and provided a variety of bases for seed applications as biofuel or medicine.

Keywords: *Styrax tonkinensis*; kernel development; metabolome analysis; metabolite classification

1. Introduction

*Styrax tonkinensis* (Pierre) Craib ex Hartwich, a member of the *Styracaceae* family, has high timber, medicine, oil, and ornamental value [1–3]. This species has been widely cultivated for wood production as pulpwod species and benzoin resin collection used in perfumes and medicines in Laos, Vietnam and southern China [1,4]. It has also been verified as potential for biofuel with bioethanol from woody biomass and biodiesel from seeds [5,6]. The content of seed lipid for biodiesel is dependent on the carbon balance between different nutrients and the triacylglycerol accumulation in oil bodies [3,7].
The major content of *S. tonkinensis* seeds is lipid with excellent fatty acids (FA) composition, followed by protein, soluble sugar and amino acids. *S. tonkinensis* seed oil has perfect biodiesel properties [6], satisfying the standards of China (GB/T 20828), the European Union (EN 14214), Germany (DIN V51606) and the USA (ASTM D6751). However, despite the studies focusing on the functional potential of *S. tonkinensis* seeds, it has not been given enough attention to the knowledge of the comprehensive biochemical composition.

Plant seed, as an embryonic plant formed on the mother tree, is very important for the reproduction of spermatophytes. Seed formation is determined by a series of metabolic processes of nutrient accumulation related to sugar, starch, lipid, amino acids, protein and other secondary metabolites with large species-specific difference [8–10]. There are many important metabolic pathways involved in seed development, e.g., amino acids metabolism, starch and sucrose metabolism, FA and flavonoids biosynthesis [11,12]. Understanding the active pathways involved in *S. tonkinensis* seed development requires approaches of metabolomics.

Metabolomics, as an alternative to genomics, transcriptomics and proteomics, plays an important role in studying the relationship between genotypes and phenotypes through related analyses of metabolites and pathway enrichment during plant development [13]. Metabolite fingerprinting can be conducted with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), the former approach is widely used for analyzing volatile organics and the latter for larger, more polar molecules [14,15]. Therefore, active metabolites and related pathways have been studied to assess metabolism levels in developing seeds, such as *Thlaspi arvense* L. and *Dipteryx alata* Vog. [16,17], which can partially provide the basis for studying in *S. tonkinensis*.

In this work, we analyzed samples from four stages during *S. tonkinensis* kernel development to evaluate metabolite fingerprinting and pathway enrichment by both GC-MS and LC-MS. The methodology used allowed us to understand the biochemical basis for developing *S. tonkinensis* kernels by (i) identifying metabolites related to main nutritional components biosynthesis, (ii) performing classification and change trends of identified metabolites, and (iii) confirming major metabolic pathways based on differential metabolites identification and pathway enrichment in developing kernels.

2. Results

2.1. Quality Control Result

The samples from 50, 70, 100 and 130 days after flowering (DAF) were analyzed through the GC-MS and LC-MS methods. Total ion currents were obtained after metabolomics analyses. These mass spectrograms were uniform at chromatographic peak, abundant in number and good in shape, without undesirable phenomena such as over-saturation or tail dragging which indicated good methods and the high quality of samples. The obtained metabolomics data of the GC-MS and LC-MS analyses adhered to a normal distribution, and a subsequent principal component analysis (PCA) showed high repeatability across biological replicates, as samples from four time points were distributed into four regions in a counter-clockwise sense except one from 100 DAF in LC-MS analysis (Figure 1). Hierarchical cluster analysis (HCA) showed similar result to the PCA analysis, with all of the eight biological replicates from each time point being clustered together (Figure S1).
with downward trends during all kernel development with a 10-day phase in the last stage or middle stage. Group VIII contained 64 metabolites, representing metabolites with lower abundant levels in the late stage (100 and 130 DAF) than in the early stage (50 and 70 DAF).

2.2. Metabolite Identification

A total of 187 metabolites were identified by GC-MS analysis in developing S. tonkinensis kernels (Table S1), which were grouped into 19 classes (Figure 2a). There were 54 metabolites (28.88%) classified into carboxylic acids and derivatives class, which mainly contained amino acids. The total amino acid concentration showed an up-down change trend peaking at 100 DAF. Alanine, glutamine, tryptophan, tyrosine and valine were the five most abundant amino acids across S. tonkinensis kernel development, representing between 74% and 95% of the total amino acids. Fifty metabolites (26.74%) were clustered into organooxygen compounds class with the major sub-class of the carbohydrates and carbohydrate conjugates, following the alcohols and polyols. Sucrose and glucose were the main soluble sugars, while inositol was the main sugar alcohol in developing S. tonkinensis kernels. Fatty acyls and benzene and substituted derivatives classes had 11 metabolites (5.88%), respectively. These 187 metabolites were clustered into eight groups based on their change patterns (Figure 3a). Groups I, II, III, and VI containing 19, 21, 20 and 25 metabolites, respectively, represented the metabolites with peak abundant levels at 50, 70, 100, and 130 DAF. Group V contained 13 metabolites with low abundant levels at 70 DAF. Groups VI and VII, containing 13 and 12 metabolites, respectively, represented metabolites with downward trends during all kernel development with a 10-day phase in the last stage or middle stage. Group VIII contained 64 metabolites, representing metabolites with lower abundant levels in the late stage (100 and 130 DAF) than in the early stage (50 and 70 DAF).

---

**Figure 1.** Quality control of samples from biological material. (a) Principal component analysis (PCA) of gas chromatography-mass spectrometry (GC-MS) analysis. (b) PCA of liquid chromatography-mass spectrometry (LC-MS) analysis.

**Figure 2.** A classification of the metabolites during developing S. tonkinensis kernels. (a) The clustering results based on the metabolites of GC-MS analyses. (b) The clustering results based on the metabolites of LC-MS analyses.
Metabolites 2020, 10, 21

Figure 3. The K-means clustering of the metabolites during developing S. tonkinensis kernels. (a) The clustering results based on the metabolites of GC-MS analyses. (b) The clustering results based on the metabolites of LC-MS analyses.

A total of 1556 metabolites were identified by LC-MS analysis in developing S. tonkinensis kernels (Table S2), which were grouped into 21 classes (Figure 2b). The most abundant class was the flavonoids class (225 metabolites, 14.37%), followed by the fatty acyls (222 metabolites, 14.18%), glycerophospholipids (130 metabolites, 8.30%), steroids and steroid derivatives (127 metabolites, 8.11%) and prenol lipids (115 metabolites, 7.34%). The metabolites in flavonoids class were mainly formed as flavans (FV, e.g., glicoisoflavanone), flavonoid glycosides (FG, e.g., quercetin 3, 7, 4′-O-triglucoside) and o-methylated flavonoids (OMF, e.g., 3, 4′, 5-trihydroxy-3′, 7-dimethoxyflavanone), which peaked at 50, 70 and 100 DAF, respectively. Among them, FG, composed of most kinds of metabolites, accounted for the major proportion but with a downward trend (dropping from 84.08% to 43.67%) during S. tonkinensis kernel development. Besides, OMF showed an upward trend from 9.95% to 30.20%, while FV increased from 2.40% to 23.70%. For fatty acyls class, there were four major free FAs, i.e., palmitic acid (C16:0), stearic acid (C18:0), oleic acid (18:1) and linoleic acid (C18:2). The FAs of C16:0 and C18:0 showed downward trends, while C18:1 and C18:2 had upward trends during S. tonkinensis kernel development. There were nine sub-classes in the glycerophospholipids class, i.e., cardiolipin (CL), cyclic lysophosphatidic acid (CPA), lysophospholipid (LyP), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyglycerol (PG), phosphatidylserine (PS) and phosphatidylserine (PS). Among them, LyP accounted for the largest proportion with a down-up change trend, following PI with an up-down trend. The change in steroids and steroid derivatives class showed a downward trend overall during S. tonkinensis kernel development, with a major sub-class of steroidal glycosides. The content of prenol lipid remained stable with three major sub-classes of diterpenoids, monoterpenoids, and terpene glycosides. According to
their change trends, these 1556 metabolites were classified into eight groups (Figure 3b). Clusters I, II, III, and IV contained 241, 147, 124 and 166 metabolites, respectively, representing the metabolites with peaking abundances at 50, 70, 100, and 130 DAF. Cluster V, containing 188 metabolites, represented metabolites that showed similar change trends to metabolites in group VI, having continuous downward trends throughout kernel development. Cluster VI contained 193 metabolites, representing metabolites with lower levels at the initial stage. Cluster VII, containing 238 metabolites, represented metabolites with high expression levels in the late stage (100 and 130 DAF). Cluster VIII, containing 269 metabolites, represented metabolites having somewhat lower expression levels in the late stage (100 and 130 DAF) than in the early stage (50 and 7 DAF).

For the classification and clustering of different metabolites (Tables S1 and S2), carboxylic acids and derivatives were identified by both LC-MS (100) and GC-MS (54), as they had similar change trends with high levels in late stages within group VIII and cluster VII. Organooxygen compounds were identified by both LC-MS (106) and GC-MS (50), and their abundance showed high levels in the early stage (group I and cluster I) or late stage (group VIII and cluster VII). Benzene and substituted derivatives were mainly identified through GC-MS (11), showing high levels in later stages (100 and 130 DAF) within group VIII. Flavonoids were mostly identified through LC-MS, and their abundance showed many obvious change trends within clusters I (45), IV (41), VI (33) and VII (37). Fatty acyls were also mainly identified by LC-MS, and had high levels in early stages within clusters I (32), II (26), III (27), V (30), and VIII (46). And, glycerophospholipids (130) and steroids and steroid derivatives (127) were mainly identified by LC-MS.

2.3. Differential Metabolites Analysis and Pathway Enrichment

Differential metabolites (DMs) between different time points were selected according to a VIP > 1 and a p-value < 0.05. Relative to each precious sampling point in GC-MS, a total of 72, 93, and 78 DMs were identified at 70, 100 and 130 DAF, respectively (Figure 4a). Relative to 50 DAF, a total of 101 and 103 DMs were identified at 100 and 130 DAF, respectively. There were 99 DMs identified between 70 and 130 DAF. For Venn diagrams of GC-MS analysis (Figure 4b), there were 56 DMs identified between the early stage (50 and 70 DAF) and late stage (100 and 130 DAF), and most of the DMs were upregulated. As same with the standard of GC-MS, DMs in LC-MS were identified to investigate changes in metabolism profile. Relative to each precious sampling point, a total of 208, 186 and 160 DMs were identified at 70, 100 and 130 DAF, respectively (Figure 4c). Relative to 50 DAF, a total of 211 and 224 DMs were identified at 100 and 130 DAF, respectively, while there were 182 DMs identified between 70 and 130 DAF. For Venn diagrams of LC-MS analysis (Figure 4d), the DMs were mainly concentrated (102 of the 358 DMs) between the early stage (50 and 70 DAF) and late stage (100 and 130 DAF), and the number of upregulated and downregulated was little different.

Based on the DMs identified during S. tonkinensis kernel development, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was analyzed (Table S3). Alanine, aspartate and glutamate metabolism (ath00250), arginine and proline metabolism (ath00330), and glutathione metabolism (ath00480) were enriched between all sampling points of the DMs of both GC-MS and LC-MS analyses. Butanoate metabolism (ath00650), glycerolipid metabolism (ath00561), nitrogen metabolism (ath00910), the TCA cycle (ath00020), the pentose phosphate pathway (PPP) (ath00030), and pyrimidine metabolism (ath00240) were mainly enriched between all sampling points by DMs of GC-MS analysis. Biosynthesis of unsaturated fatty acids (ath01040), carbon fixation in photosynthetic organisms (ath00710), diterpenoid biosynthesis (ath00904), fatty acid biosynthesis (ath0061), flavonoid biosynthesis (ath00941), riboflavin metabolism (ath00740), and starch and sucrose metabolism (ath00500) were mainly enriched between the early stages (50 and 70 DAF) and late stages (100 and 130 DAF) of the LC-MS analysis.
A similar trend was also found in LS-MS analysis, which was consistent with the rapid increase in the processes during plant development [18]. However, it needs effective quality control with complex multi-component samples to avoid the significant problems posed by unknown components [19]. In our study, we measured the change trends of metabolites in developing kernels through two metabolomics approaches, i.e., LC-MS and GC-MS analyses. The PCA and HCA showed high repeatability across biological replicates [20], with replicates from every time point being clustered together. In the PCA analysis, dim 1 may have been related to the time of kernel development from 50 DAF to 130 DAF, while dim 2 may have been related to the metabolic activity, with the beginning of nutrition accumulation around 70 DAF during S. tonkinensis kernel development [21].

During seed development, nutrients are transported into seeds and stored as nutritional components, e.g., amino acids, soluble protein, lipid, soluble sugar and starch [10]. Amino acids are important and active in developing kernels and are easily identified by GC-MS analysis [22]. The total amino acid content analyzed in GC-MS analysis showed an up-down trend with peaking at 100 DAF.

A similar trend was also found in LS-MS analysis, which was consistent with the rapid increase in the total soluble protein content during kernel development [3]. Among the identified FAs, there were two major unsaturated FAs, i.e., C18:1 and C18:2, which showed continuously increasing trends and high proportions, with similar results in developing Arachis hypogaea L. kernels [23]. A high degree of unsaturated FAs reflects good fuel characteristics for bioresources [24], including density (\(\rho\)), kinematic viscosity (\(\eta\)), cetane number (CN), iodine value (IV), and cold filter plugging point (CFPP), which were analyzed in developing S. tonkinensis kernels by Wu et al. [6]. LyP, as an intermediate of triacylglycerol
(TAG) biosynthesis [25], was the main form of metabolites in glycerophospholipid class (about 50%) with two major contents of lyP(18:1(9Z)) and lyP(18:2(9Z,12Z)). The main intracellular sugars and amino acids were found to be sucrose, glucose, tyrosine and glutamine, which were respectively common sources of carbon and nitrogen for developing plant embryos [17]. Furthermore, some phosphorylated metabolites (as key intermediates of glycolysis) of the PPP and TCA cycle (which is related to carbon metabolism) were identified, e.g., fructose-6-phosphate, fructose-1,6-bisphosphate, trehalose-6-phosphate and UDP-glucose [26], which showed high abundance levels at 70 and 100 DAF with rapid increases in lipid and starch biosynthesis during S. tonkinensis kernel development [21].

As bark produces the abundant benzoin used in incense, perfumes and medicines [27], S. tonkinensis has high economic and medicinal value [28,29] with abundant secondary metabolites. Flavonoids, as important plant secondary metabolites, are present in plant fruits, leaves and roots, such as Polygonum minus Huds. and Mauritia flexuosa L. [29,30]; and they can be found in many natural products beneficial to human health, such as teas and wines [31]. However, there are no studies that have focused on the secondary metabolites in S. tonkinensis kernels. In this study, we found 225 kinds of flavonoids through LC-MS in developing S. tonkinensis kernels. Among the flavonoids class, three main sub-classes, i.e., FG, FV and OMF accounted for up to 97.57% of flavonoids at 130 DAF, which could improve biological activities and pharmacological properties [32,33]. Otherwise, we found some metabolites classified into steroids and steroids derivatives, but the content decreased continuously to only 1/20 that of flavonoids. The identification of a high abundance of secondary metabolites in developing S. tonkinensis kernels may confirm that the species seeds have high medicinal value as its bark, like benzoin resin [1,34,35].

Metabolomics is an effective tool to assess the metabolites’ state during plant development [36]. Pathway analysis based on metabolites can provide insight into the complex biological processes of plant biomass biosynthesis, e.g., amino acids, organic acids, soluble sugar and protein [37]. In our study, the pathway enrichment mainly focused on three groups, such as pathways related to amino acids metabolism, carbon flow from sucrose to lipid and secondary metabolites biosynthesis, which are indications of protein, oil, and flavonoid accumulation in developing kernels, respectively [38,39]. Pathways related to amino acid metabolism were complex and involved in the biosynthesis of various kinds of amino acids, which play important roles in cellular processes [40]. Pathways related to carbon flow from sucrose to lipid, including glycolysis, the TCA cycle, the PPP, and FA biosynthesis, affect lipid accumulation in oilseeds [26]. The active pathway enrichment involved in amino acids and carbon flow was consistent with that protein (20%) and lipid (60%) are main nutrients in S. tonkinensis kernels, with perfect biodiesel properties for oil-seed resource [21]. Lipid content has a 40-day decrease during S. tonkinensis kernel development, which was considered as the result of carbon competition between oil and starch by Zhang et al. [3], but there is no effective evidence. Moreover, there are some gaps in the term of the total dry matter that needs to be studied, e.g., flavonoids and prenol lipid. The presence of various kinds of flavonoids and active pathway enrichment indicated that secondary metabolites account for a certain proportion in developing S. tonkinensis kernels and then may lead to a decrease in carbon flux to lipid around 100 DAF.

We analyzed the comprehensive biochemical composition by sampling at 50, 70, 100, and 130 DAF. The size and structure of S. tonkinensis kernel began to develop at 50 DAF, and lipid began to appear. Most nutrients in the kernels began to accumulate rapidly at 70 DAF with high enzyme activity, e.g., acetyl-CoA carboxylase, phosphoglucose isomerase, and the pyruvate dehydrogenase complex [3,21]. There was a continuously decreasing trend in lipid content between 80 and 120 DAF, which may have been related to the biosynthesis of flavonoids analyzed in the present study rather than the carbon competition between oil and starch. The seeds matures at 130 DAF, which is close to the best harvest time to get S. tonkinensis seed oil of better quantity and quality for biodiesel. Overall, the time points selected in this study represented four important turning points of kernel development, nutrients accumulation, flavonoids accumulation and kernel harvest.
4. Materials and Methods

4.1. Plant Materials

Forty *S. tonkinensis* plants were used in this study, which were planted in the Styracaceae Germplasm Repository located in the Luhe District of Nanjing, China (32°32′ N, 118°50′ E). Trees flowered in late May and were tagged for sampling later. According to our previously published data [3,21] on the morphological and physiological changes of developing *S. tonkinensis* kernels, we selected samples from four time points in order to explore changes in the metabolome, i.e., 50 DAF (the previous stage before seed matter rapid increase), 70 DAF (during the aforementioned steep rise in nutrient content), 100 DAF (the stage with decreasing oil content and increasing starch content) and 130 DAF (the final maturation stage). Fresh fruits were randomly collected and immediately transferred to the laboratory on dry ice with eight biological replicates at each time point. The pericarp and seed coat were removed, and the kernels were immediately frozen in liquid nitrogen and stored at −80 °C for metabolomics analysis.

4.2. GC-MS Analysis

4.2.1. GC-MS Sample Preparation

1. 60 mg samples were transferred into a 1.5 mL Eppendorf tube with two small steel balls.
2. 40 µL boc-2-chloro-L-phenylalanine in 0.3 mg/mL methanol solution as internal standard and 360 µL pre-cooling methanol were added respectively.
3. The samples were placed at −80 °C for 2 min and then ground at 60 Hz for 2 min, before ultrasonic extraction at room temperature for 30 min.
4. 200 µL chloroform and 400 µL water were added.
5. The samples were extracted at room temperature for 30 min and then centrifuged at 12,000 rpm at 4 °C for 10 min.
6. 500 µL supernatant was transferred into GC vials.
7. The samples were dried with a freeze concentration centrifugal dryer, and then 80 µL methoxamine-hydrochloride in pyridine (15 mg/mL) was added (with vortex oscillation for 2 min). This was placed at 37 °C for 90 min for an oximation reaction.
8. 80 µL bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and 20 µL n-hexane were added, and vortex oscillation for 2 min followed.
9. The samples were placed at 70 °C for 60 min and then at room temperature for 30 min for GM-LC analysis.
10. Quality control samples (QCs) were prepared by mixing all samples, which were analyzed using the same method as with the analytic samples. The QCs were injected at regular intervals to assess the repeatability of the whole analytical process.

4.2.2. GC Conditions and MS Method

The GC-MS analysis was performed on an Agilent 7890A/5975C GC-MS system (Agilent, Santa Clara, CA, USA) equipped with two split/-splitless inlets and a multi-purpose sampler using a HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent J&W Scientific, Folsom, CA, USA). Refer to previous studies [17,41] for the appropriate modifications during the separation process.

The injection volume of the sample was 1 µL and the injector temperature was 260 °C. The carrier gas was helium at a flow of 1 mL/min. The GC temperature was initially 60 °C for 2 min, and then it gradually increased at 8 °C/min to 310 °C, which was held for 6 min.

An electro ionization system was used with 70-eV of ionization energy. The mass spectra of eluted compounds were recorded over an *m/z* range of 50–600.
4.2.3. GC-MS Data Collection

The GC-MS raw data were analyzed by Chroma TOF v4.34 software (LECO, Stockport, UK). After removing the internal standard peak and any known false positive peaks, e.g., including noise, column loss and derivative peaks, the GC-MS data were obtained with three-dimensional datasets including sample information, metabolism names, and peak intensities.

4.3. LC-MS Analysis

4.3.1. LC-MS Sample Preparation

1. 80 mg samples were transferred into a 1.5 mL Eppendorf tube with two small steel balls.
2. 20 µL boc-2-chloro-L-phenylalanine in methanol and water (1/1, v/v) as internal standard and 1 mL pre-cooling methanol solution (7/1, v/v) were added respectively.
3. The samples were placed at −80 °C for 2 min and then ground at 60 Hz for 2 min, before ultrasonic extraction at room temperature for 30 min.
4. The samples were placed at 4 °C for 20 min and then centrifuged at 14,000 rpm at 4 °C for 10 min.
5. 500 µL supernatants were filtered through 0.22 µm microfilters and then transferred into LC vials.
6. QCs were prepared by mixing all of the all samples in the same way as with the GC-MS analysis.

4.3.2. LC Conditions and MS Method

The LC-MS analysis was performed on an Ultimate 3000-Velos Pro system (Dionex, Sunnyvale, CA, USA) equipped with a binary solvent delivery manager and a multi-purpose sampler, coupled with a Q exactive orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Refer to previous studies [42] for the appropriate modifications during the separation process.

The LC separation was performed using a BEH C18 column (100 nm × 2.1 mm I.D., 1.7 µm; Waters, Milford, CT, USA) at a flow rate of 0.4 mL/min and a column temperature of 45 °C.

During the LC process, mobile phase A was water with 0.1% formic acid (v/v) and mobile phase B was acetonitrile containing 0.1% formic acid (v/v).

The separation was performed using the following gradient: going from 5% to 25% B in 1.5 min, from 25% to 100% in 8.5 min, at 100% for 3 min, then declining from 100% to 5% in 0.5 min and at 5% for 1 min.

The MS data were collected using an LTQ Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source operated in either positive or negative ion mode. The capillary and source temperature were set at 350 °C, with a desolvation gas flow of 45 L/h. Centroid data were collected from 50 m/z to 1000 m/z with a 30,000 resolution.

4.3.3. LC-MS Data Collection

The LC-MS raw data were analyzed by Progenesis QI v2.0 software (Waters Corporation, Milford, CT, USA) with a retention time (RT) range of 0.5–14 min, a mass range of 50–1000 Da, and a mass tolerance of 0.01 Da. Isotopic peaks were excluded from the analysis, the noise elimination level was set at 10, the minimum intensity was set to 15% of base peak intensity, and finally, the RT tolerance was set at 0.01 min. The LC-MS data were obtained with three-dimensional datasets included m/z, peak RT, and peak intensities, and RT-m/z pairs were used as the identifier for each ion.

4.4. Data Analysis and Bioinformatics Analysis

The GC-MS and LC-MS data were imported into the SIMCA-P + 14.0 software package (Umetrics, Umeå, Sweden) for statistical analysis [37]. A PCA, HCA and (orthogonal) partial least-squares-discriminant analysis ((O)PLS-DA) were carried out to visualize the metabolic alterations among experimental groups, after mean centering and unit variance scaling. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the (O)PLS-DA model, and those
variables with VIP > 1.0 were considered relevant for group discrimination. In this study, a default seven-round cross-validation was applied, with one-seventh of the samples being excluded from the mathematical model in each round in order to guard against overfitting. The identified metabolites were grouped by their biochemical properties and change patterns (www.omicsolution.org). The differential metabolisms were identified by (O)PLS-DA if their VIP was over 1 with a p-value < 0.05. And then the pathway enrichment according to the identified differential metabolisms was analyzed using KEGG databases [43].

5. Conclusions

In our study, we collected samples from 50, 70, 100, and 130 DAF to explore spatiotemporal comprehensive biochemical composition. It’s the first time to identify flavonoids class in S. tonkinensis kernels, which were mainly formed as flavans, flavonoid glycosides and o-methylated flavonoids. All of the metabolites were grouped based on their biochemistry properties and change trends. The major metabolite classes were focused on carboxylic acids and derivatives, flavonoids, fatty acyls, glycerophospholipids, organooxygen compounds, prenol lipids, and steroids and steroid derivatives. Alanine, glutamine, tryptophan, tyrosine, and valine were the top five most abundant amino acids; and palmitic acid, stearic acid, oleic acid, and linoleic acid were four major free FAs. Pathway enrichment was mainly focused on three groups, amino acids metabolism, carbon flow from sucrose to lipid and secondary metabolites biosynthesis. S. tonkinensis seeds have application value in biofuels and medicines with high contents lipid and flavonoids.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-1989/10/1/21/s1, Figure S1: The results of the hierarchical cluster analysis (HCA) in the GC-MS and LC-MS analyses. (a) HCA analysis in GC-MS, (b) HCA analysis in LC-MS, Table S1: The list of metabolites identified by GC-MS analysis, Table S2: The list of metabolites identified by LC-MS analysis, Table S3: The pathway enrichment analysis based on differential metabolites.

Author Contributions: Conceptualization, F.Y. and Q.W.; methodology, Q.W., X.Z. and C.C.; formal analysis, Q.W., X.Z., C.C. and Z.Z.; funding acquisition, F.Y.; data curation, Q.W. and Z.Z.; writing—original draft preparation, Q.W.; writing—review and editing, Q.W., Z.Z. and F.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by “A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD)”, the National Natural Science Foundation of China (3197140894) and the Doctorate Fellowship Foundation of Nanjing Forestry University.

Acknowledgments: We thank Xiaoyue Ji from Advanced Analysis Testing Center, Nanjing Forestry University for technical assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

References
1. Pinyopusarerk, K. Styrax tonkinensis: Taxonomy, ecology, silviculture and uses. In ACIAR Technical Reports No.31, 2nd ed.; Julie, H., Ed.; CPN Publication: Canberra, Australia, 1994; Volume 20, pp. 1–20.
2. Xu, L.P.; Yu, F.Y. Corolla structure and fragrance components in Styrax tonkinensis. Trees Struct. Funct. 2015, 29, 1127–1134. [CrossRef]
3. Zhang, Z.H.; Wang, X.J.; Luo, Y.; Yu, F.Y. Carbon competition between fatty acids and starch during benzoin seeds maturation slows oil accumulation speed. Trees Struct. Funct. 2017, 31, 1025–1039. [CrossRef]
4. Courel, B.; Adam, P.; Schaeffer, P. The potential of triterpenoids as chemotaxonomic tools to identify and differentiate genuine, adulterated and archaeological balsams. Microchem. J. 2019, 147, 411–421. [CrossRef]
5. Sasaki, C.; Negoro, H.; Asada, C.; Nakamura, Y. Microwave-assisted glucose production from bode (Styrax tonkinensis) woody biomass for bioethanol production. J. Mater. Cycles Waste Manag. 2019, 21, 201–204. [CrossRef]
6. Wu, Q.K.; Fei, X.R.; Gao, Y.; Chen, C.; Cao, Y.Y.; Yu, F.Y. Comparative analysis of fuel properties of eight biodiesel plants species of Styrax spp. China Oils Fats 2019, 44, 27–30.
Zhang, Z.H.; Luo, Y.; Wang, X.J.; Yu, F.Y. Quantitative spatiotemporal oil body ultrastructure helps to verify the distinct lipid deposition patterns in Benzoix endosperm and embryo cells. *Forests* 2018, 9, 265. [CrossRef]

Zhang, C.X.; Tanabe, K.; Tamura, F.; Matsumoto, K.; Yoshida, A. 13C-photosynthate accumulation in Japanese pear fruit during the period of rapid fruit growth is limited by the sink strength of fruit rather than by the transport capacity of the pedicel. *J. Exp. Bot.* 2005, 56, 2713–2719. [CrossRef]

Conde, C.; Delrot, S.; Geros, H. Physiological, biochemical and molecular changes occurring during oliv development and ripening. *J. Plant Physiol.* 2008, 165, 1545–1562. [CrossRef]

Ruan, Y.L.; Patrick, J.W.; Bouzayen, M.; Orsorio, S.; Ferrie, A.R. Molecular regulation of seed and fruit set. *Trends Plant Sci.* 2012, 17, 656–665. [CrossRef]

Hajduch, M.; Casteel, J.E.; Hurrelmeyer, K.E.; Song, Z.; Agrawal, G.K.; Thelen, J.J. Proteomic analysis of seed filling in *Brassica napus*. developmental characterization of metabolic isozymes using high-resolution two-dimensional gel electrophoresis. *Plant Physiol.* 2006, 141, 32–46. [CrossRef]

Matheus, M.T.; Lopes, J.C.; Correa, N.B. Physiological maturation of *Erythrina variegata* L. seeds. *Cienc. Florest.* 2011, 21, 619–627. [CrossRef]

Ogura, T.; Bamba, T.; Fukusaki, E. Development of a practical metabolite identification technique for non-targeted metabolomics. *J. Chromatogr. A* 2013, 1301, 73–79. [CrossRef]

Lisec, J.; Schauer, N.; Kopka, J.; Willmitzer, L.; Ferrie, A.R. Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat. Protoc.* 2006, 1, 387–396. [CrossRef]

Allwood, J.W.; Goodacre, R. An introduction to liquid chromatography-mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochem. Anal.* 2010, 21, 33–47. [CrossRef]

Marques, F.G.; Neto, J.R.D.; da Cunha, L.C.; de Paula, J.R.L.; Bara, M.T.F. Identification of terpenes and phytosterols in *Diperyx alata* (baru) oil seeds obtained through pressing. *Rev. Bras. Farmacogn. Braz.* J. 2015, 25, 522–525. [CrossRef]

Tsogbaataar, E.; Cocuron, J.C.; Sonera, M.C.; Alonso, A.P. Metabolite fingerprinting of pennycress (*Thlaspi arvense*) embryos to assess active pathways during oil synthesis. *J. Exp. Bot.* 2015, 66, 4267–4277. [CrossRef]

Theodoridis, G.A.; Gika, H.G.; Want, E.J.; Wilson, I.D. Liquid chromatography-mass spectrometry based global metabolite profiling: A review. *Anal. Chim. Acta* 2012, 711, 7–16. [CrossRef]

Sangster, T.; Major, H.; Plumb, R.; Wilson, A.J.; Wilson, I.D. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabolonomic analysis. *Analyst* 2006, 131, 1075–1078. [CrossRef]

Gherardt, N.; Schwolow, S.; Rohn, S.; Perez-Cacho, P.R.; Galan-Soldevilla, H.; Arce, L.; Weller, P. Quality assessment of olive oils based on temperature-ramped HS-GC-IMS and sensory evaluation: Comparison of different processing approaches by LDA, kNN, and SVM. *Food Chem.* 2019, 278, 720–728. [CrossRef]

Wu, Q.K.; Zhang, Z.H.; Peng, H.; Wu, Y.L.; Yu, F.Y. The nutrient distribution in the continuum of the pericarp, seed coat, and kernel during *Stylosia tonkinensis* fruit development. *PeerJ* 2019, 7, e7996. [CrossRef]

Alonso, A.P.; Val, D.L.; Shachar-Hill, Y. Central metabolic fluxes in the endosperm of developing maize seeds and their implications for metabolic engineering. *Metab. Eng.* 2011, 13, 96–107. [CrossRef] [PubMed]

Liu, H.; Li, H.F.; Gu, J.Z.; Deng, L.; Ren, L.; Hong, Y.B.; Lu, Q.; Chen, X.P.; Liang, X.Q. Identification of the candidate proteins related to oleic acid accumulation during peanut (*Arachis hypogaea*) seed development through comparative proteome analysis. Int. J. Mol. Sci. 2018, 19, 1235. [CrossRef] [PubMed]

Wang, L.B.; Yu, H.V.; He, X.H.; Liu, R.Y. Influence of fatty acid composition of woody biodiesel plants on the fuel properties. *J. Fuel Chem. Technol.* 2012, 40, 397–404. [CrossRef]

Xu, C.C.; Shanklin, J. Triacylglycerol metabolism, function, and accumulation in plant vegetative tissues. *Annu. Rev. Plant Biol.* 2016, 67, 179–206. [CrossRef] [PubMed]

Rawsthorne, S. Carbon flux and fatty acid synthesis in plants. *Prog. Lipid Res.* 2002, 41, 182–196. [CrossRef]

Burger, P.; Casale, A.; Kerdudo, A.; Michel, T.; Laville, R.; Chagnaud, F.; Fernandez, X. New insights in the chemical composition of benzoin balsams. *Food Chem.* 2016, 210, 613–622. [CrossRef]

Moore, B.D.; Andrew, R.L.; Kulheim, C.; Foley, W.J. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytol.* 2014, 201, 733–750. [CrossRef]

Leke, K.K.; Rahnamalie-Tajadod, R.; Yeoh, C.C.; Goh, H.H.; Mohamed-Hussein, Z.A.; Zainal, Z.; Ismail, I.; Noor, N.M. Transcriptome analysis of *Polygonum minus* reveals candidate genes involved in important secondary metabolic pathways of phenylpropanoids and flavonoids. *PeerJ* 2017, 5, e2938. [CrossRef]
30. Nonato, C.D.A.; Leite, D.O.D.; Pereira, R.C.; Boligon, A.A.; Ribeiro, J.; Rodrigues, F.F.G.; da Costa, J.G.M. Chemical analysis and evaluation of antioxidant and antimicrobial activities of fruit fractions of Mauritia flexuosa L. f. (Arecaeeae). *PeerJ* **2018**, *6*, e5991. [CrossRef]

31. Agrawal, A.D. Pharmacological activities of flavonoids: A review. *Int. J. Pharm. Sci. Nanotechnol.* **2011**, *6*, 1394–1398.

32. Koirala, N.; Thuan, N.H.; Ghimire, G.P.; Thang, D.V.; Sohng, J.K. Methylation of flavonoids: Chemical structures, bioactivities, progress and perspectives for biotechnological production. *Enzyme Microb. Technol.* **2016**, *86*, 103–116. [CrossRef] [PubMed]

33. Li, J.; Hossain, M.S.; Ma, H.C.; Yang, Q.H.; Gong, X.W.; Yang, P.; Feng, B.L. Comparative metabolomics reveals differences in flavonoid metabolites among different coloured buckwheat flowers. *J. Food Compos. Anal.* **2020**, *85*, 103335. [CrossRef]

34. Havsteen, B.H. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **2002**, *96*, 67–202. [CrossRef]

35. Feng, L.; Zhai, Y.Y.; Xu, J.; Yao, W.F.; Cao, Y.D.; Cheng, F.F.; Bao, B.H.; Zhang, L. A review on traditional uses, phytochemistry and pharmacology of Eclipta prostrata L. *J. Ethnopharmacol.* **2019**, *245*, 1–14. [CrossRef] [PubMed]

36. Patti, G.J.; Yanes, O.; Siuzdak, G. Metabolomics: The apogee of the omics trilogy. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 263–269. [CrossRef] [PubMed]

37. Li, X.R.; Pei, G.S.; Liu, L.S.; Chen, L.; Zhang, W.W. Metabolomic analysis and lipid accumulation in a glucose tolerant *Crypthecodinium cohnii* strain obtained by adaptive laboratory evolution. *Bioresour. Technol.* **2017**, *235*, 87–95. [CrossRef] [PubMed]

38. Simcox, P.D.; Dennis, D.T. Isoenzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of *Ricinus communis* L. *Plant Physiol.* **1978**, *61*, 87–877. [CrossRef]

39. Routaboul, J.M.; Kerhoas, L.; Debeaujon, I.; Pourcel, L.; Caboche, M.; Einhorn, J.; Lepiniec, L. Flavonoid diversity and biosynthesis in seed of *Arabidopsis thaliana*. *Planta* **2006**, *224*, 86–107. [CrossRef]

40. Meng, X.X.; Mujahid, H.; Zhang, Y.D.; Peng, X.J.; Redona, E.D.; Wang, C.L.; Peng, Z.H. Comprehensive analysis of the lysine succinylome and protein co-modifications in developing rice seeds. *Mol. Cell. Proteom.* **2019**, *18*, 2359–2372. [CrossRef]

41. Mokhetho, K.C.; Sandasi, M.; Ahmad, A.; Kamatou, G.P.; Viljoen, A.M. Identification of potential anti-quorum sensing compounds in essential oils: A gas chromatography-based metabolomics approach. *J. Essent. Oil Res.* **2018**, *30*, 399–408. [CrossRef]

42. Tian, L.; Perot, S.J.; Stevenson, D.; Jacobson, T.; Lanahan, A.A.; Amador-Noguez, D.; Olson, D.G.; Lynd, L.R. Metabolome analysis reveals a role for glyceraldehyde 3-phosphate dehydrogenase in the inhibition of *C. thermocellum* by ethanol. *Biotechnol. Biofuels* **2017**, *10*, 276. [CrossRef] [PubMed]

43. Ogata, H.; Goto, S.; Sato, K.; Fujibuchi, W.; Bono, H.; Kanehisa, M. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **1999**, *27*, 29–34. [CrossRef] [PubMed]