Color for Life: Biosynthesis and Distribution of Phenolic Compounds in Pepper (*Capsicum annuum*)

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Abstract: Fruits and vegetables are an important supplier of biological active substances, such as vitamins and secondary metabolites (SM) for human nutrition, but also for further industrial applications. Pepper (*Capsicum annuum*) is grown and consumed all over the world as a fresh vegetable or dried as a spice. It is also used as a coloring agent, as well for medical purposes. Pepper fruits are considered as an attractive source of health-related compounds, but other organs like the leaves and stem also contain considerable amounts of antioxidants, e.g., phenolic compounds. This indicates potential for valorization of residual biomass from horticultural production by using innovative bioeconomic concepts. Herein, we present an overview about the biosynthesis of phenolic compounds, with a special focus on flavonoids and their regulation in pepper, the current knowledge of amounts and distribution of these valuable substances, as well as possible strategies for: (1) increasing flavonoid contents in pepper, (2) improving the nutritional value of fruits, and (3) new concepts for utilization of residual biomass from horticultural production.

Keywords: secondary metabolites; phenolic compounds; biosynthesis; flavonoids; human nutrition; industrial application; *Capsicum annuum*; pepper

1. Secondary Metabolites from Fruits and Vegetables are of High Value for Human Nutrition and Industrial Applications

Fruits and vegetables are an important source of phytochemicals and secondary metabolites (SM). The health-promoting properties of fruits and vegetables have long been recognized and, as a result, recommendations for daily consumption have been issued by e.g., the World Health Organization (WHO) [1] and many different countries [2,3].

Plants produce more than 200,000 different SM [4], which have no direct function in growth and maintenance of cells, but can be bio-active, and have a major role in the adaptation of plants to their environment such as protection against different forms of stresses [5,6]. For example, SM have a key role in protecting plants from herbivores and pathogens [7–10], and they can serve as UV protectants [11,12], or signaling molecules to attract pollinators [13–15].

Plant SM play an important role for human nutrition as they are significant determinants of the quality of fruits and vegetables. Some major groups of SM can be distinguished: Phenylpropanoids or phenolic compounds, terpenoids, alkaloids, and sulfuric compounds [16]. Phenolic compounds derived from plant tissues, especially flavonoids, have been shown to possess beneficial properties for human health and disease prevention [17,18], including antioxidant, antibacterial, and antiviral properties [19,20]. Protective effects have been observed for the flavonoids kaempferol, naringenin, and apigenin [21–24], and for other phenolic compounds against chronic diseases such as cardiovascular

disease, Type II diabetes, and certain cancers [25–29]. SM are also of high value for industrial applications as they represent an important source of new pharmaceutics or phytomedicines, antibiotics, cosmetics, flavors, fragrances, pesticides or fine chemicals. Plant SM are used in their natural or derivatized form or might serve as backbones for the synthesis of several valuable metabolites [30,31], e.g., approximately 60% of anticancer compounds and 75% of drugs for infectious diseases are derived from these natural products.

Peppers (*Capsicum*) are considered to have a high nutritional value due to their content of phytochemicals with known antioxidant capacity [32]. The genus *Capsicum*, comprising (non-pungent) bell peppers and chilies, belongs to the Solanaceae family and contains five domesticated species: *Capsicum annuum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Capsicum baccatum* L., and *Capsicum pubescens* Ruiz & Pav. The most varieties are found among the group of *Capsicum annuum*, which is also the economically most important species [33]. Peppers are consumed as a fresh or cooked vegetable or dried as a spice. Further, the industry uses peppers intensively as a spice or coloring agent in many foodstuffs. Peppers are grown worldwide, primarily in tropical and subtropical countries, but are also cultivated in temperate climates in greenhouses [34]. The production of peppers has been constantly increasing over the last 20 years, both in terms of cultivation area and fruit production (Figure 1A).
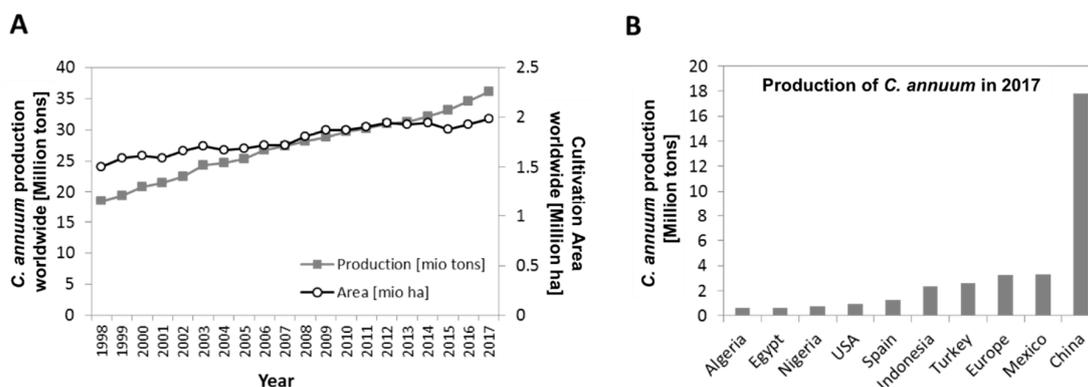
Figure 1. *Capsicum annuum* production statistics [35]. (**A**) Pepper production (grey line and squares, in million tons) and Cultivation Area (black line with white circles, in million ha) worldwide from 1998 until 2017. (**B**) Production (in million tons) of pepper in the 10 most producing countries in 2017.

In 2017, the world production of fresh peppers reached over 35 million tons of fruits harvested on almost two million ha land [35]. China was the largest producer in 2017 with more than 17 million tons of peppers, followed by Mexico, Europe, Turkey and Indonesia, who produced between one and 3.3 million tons that year (Figure 1B) [35]. Pepper fruits are an excellent source of bio-active compounds, such as vitamins, carotenoids and phenolics and have been reported to have one of the highest antioxidant capacities among vegetables [36–38]. Their suitability to tackle human micronutrient deficiency has been discussed previously [39,40].

The aim of this review is to give first an overview about the different classes of phenolic compounds and their biosynthesis, with a special focus on flavonoids. Further, the current knowledge about the phenolic composition of *Capsicum annuum* will be comprehensively reviewed and lastly, potential approaches for production of beneficial phenolic compounds in pepper, for health benefits and industrial utilization, will be discussed.

2. Phenolic Compounds—Structure and Biosynthesis

2.1. Definition and Structure of Phenolic Compounds

Phenylpropanoids and phenolic compounds originate from plant secondary metabolism. Both derive from the shikimate pathway with phenylalanine or tyrosine as intermediates [41]. They are

based on the structure of the family's central element, respectively, the aromatic or phenolic ring, often in combination with a propyl group (as it is the fact for phenylpropanoids) [42].

Based on the existing number of phenol units, phenolic compounds can either be classified as simple phenols (only one phenol unit) or polyphenols. Polyphenols consist of two or more phenolic groups, up to polymeric structures (the later will not be discussed further in this review). They have often one hydroxyl group attached to the molecule's various benzene rings [42]. In addition, polyphenols appear rarely as free compounds, but can be found in the form of esters or glycosides in plants [42,43]. They possess antioxidant properties, and their activity depends on the number and position of the hydroxyl groups and on the surrounding pH [44]. Phenolic compounds are further divided in different classes according to their chemical structure and number of carbons [42]. For example, phenolic acids have a C6-C1 basic skeleton while flavonoids have a C6-C3-C6 structure.

The polyphenolic nature of flavonoids enables them to scavenge free radicals such as superoxide and hydroxyl radicals [45]. Their C6-C3-C6 skeleton is composed of two benzene rings (ring A and B) that are linked together by three carbons (see Figure 2). It was established that ring A is located on the left side and results from the condensation of three malonyl-CoA molecules; ring B results from p-coumaroyl-CoA (from the shikimate pathway) and is located on the right side [42]. Flavonoids can be further subdivided in at least eight subgroups depending on substitutions and formation of an additional O-heterocycle (adapted from [46,47]). Many of the subgroups contain an O-heterocycle with six atoms, and only aurones have the O-heterocycle build of five atoms, while chalcones do not show any heterocyclization. In each class of flavonoids, the substitutions in the rings A and B give origin to different compounds. These substitutions may be derived from oxygenation, alkylation, glycosylation, acylation, isoprenylation and sulfonation reactions [42].
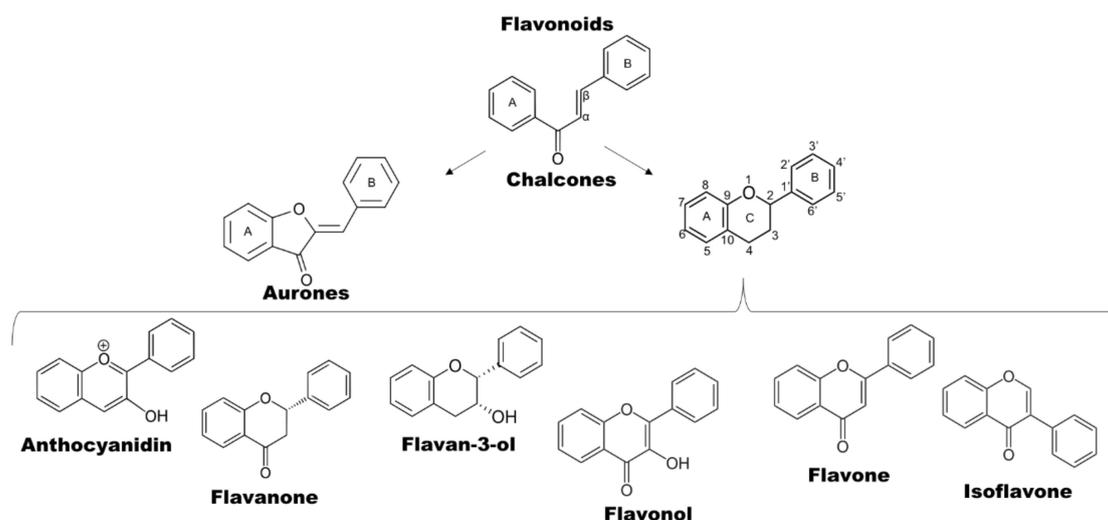
Figure 2. Subclasses of flavonoids. The central structure of flavonoids is shown for flavan, and for each subgroup the corresponding structure is shown as described in the text. Adapted from [42,46].

Chalcones are aromatic ketones based on the structure of 1-phenylethanone, in which one of the methyl hydrogens is substituted by a benzylidene group. Therefore, chalcones have two benzene rings, ring A and ring B, connected by a bridge harboring a keton conjugated to an α,β-unsaturated carbonyl (enone). In the case of dihydrochalcones, the carbon chain is saturated (no double bond). In all other subgroups this carbon bridge is part of a heterocycle. Aurones result from the cyclization of chalcones when a *meta*-hydroxyl group reacts with the α-carbon of the double bond to form a heterocycle with five atoms between ring A and B [42]. In typical flavonoids, a *meta*-hydroxy group of the ring A contributes with an atom of oxygen to the formation of a six atom-heterocycle (sometimes referred to as ring C) reacting with the β-carbon [42]. Besides chalcones and aurones, the classification of the further six subclasses depends on the substitution pattern of the aliphatic central ring C (Figure 2),

resulting in flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins [46]. These six subgroups exhibit little differences in structure, such as the degree of (un-)saturation, and/or position of substituent groups [42,43].

A high proportion of flavonoids occur naturally as water-soluble glycosides [48]. Modifications to the hydroxylation patterns in the two aromatic rings may occur, generally at the flavanone or dihydroflavonol stage, and methylation, glycosylation, and dimethylallylation are also possible, increasing the range of compounds enormously [42,49]. Dihydroflavonols are intermediates in the conversion of flavanones into flavonols, and may also be known as flavanonols [42].

2.2. Biosynthesis of Phenolic Compounds

All phenolic compounds derive from phenylalanine (or tyrosine) and its precursor shikimic acid [49]. The shikimate pathway exists only in plants and microorganisms, but not in animals (Figure 3, encircled in green). The pathway begins with the formation of 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) by DAHP synthase (DAHPS) catalyzing an aldol condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate [42,49].

Figure 3. Interplay of shikimate and phenylpropanoid pathway. A detailed description can be found in the text. *Shikimate pathway (encircled in green):* DAHPS: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DHQS: 3-dehydroquinate synthase; DHQD: 3-Dehydroquinate dehydratase; SDH: Shikimate dehydrogenase; SDK: Shikimate kinase; EPSPS: 5-Enolpyruvylshikimate-3-phosphate synthase; CS: Chorismate synthase. *Phenylpropanoid pathway (encircled in yellow):* CM: Chorismate mutase; PPA-AT: Prephenate aminotransferase; PDT: Prephenate dehydratase; ADT: Arogenate dehydratase; PPY-AT: Phenylpyruvate aminotransferase; PAL: Phenylalanine ammonia lyase; C4H: Cinnamate-4-hydroxylase; 4CL: 4-coumarate:CoA ligase (Adapted from [49–51]).

Then, phosphoric acid is removed from DAHP via 3-dehydroquinate synthase (DHQS) which further undergoes an intermolecular aldol reaction producing 3-dehydroquinic acid. Shikimic acid is formed from 3-dehydroquinic acid via 3-dehydroshikimic acid by dehydration and reduction steps both enzymatically catalyzed (via 3-dehydroquinate dehydratase (DHQD) and shikimate dehydrogenase (SDH)).

The simple phenolic acids, protocatechinic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid), can be formed from 3-dehydroshikimic acid by branch-point reactions, which involve dehydration and enolization, or, in the case of gallic acid, dehydrogenation and enolization [42,45,49]. Gallic acid may be synthetized by the same pathway as the other hydroxybenzoic

acids, although the direct aromatization of dehydroshikimic acid is considered to represent the main pathway. Gallic acid is the biosynthetic precursor structure to hydrolysable tannins and ellagic acids [42,45]. The enzymes believed to be involved mainly in this pathway are shikimate dehydrogenase and 5-dehydroshikimate dehydrogenase, although it is still under discussion whether other enzymes or pathways might be involved as well in the synthesis of gallic acid [42].

Shikimate is further converted to shikimate-3-phosphate in a phosphorylation reaction. The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) incorporates a molecule of phosphoenolpyruvate (PEP) in shikimate-3-phosphate forming 5-enolpyruvylshikimate-3-phosphate (EPSP) [49]. The latter is subsequently converted by chorismate synthase (CS) to chorismate through a 1,4-elimination of phosphoric acid.

From chorismate, phenylalanine (Phe) can be formed following the phenylpropanoid pathway, although this pathway does not belong to the shikimate pathway (Figure 3, encircled in yellow). Nevertheless, Phe is an intermediate for the phenylpropanoid pathway [42,49]. In plants, chorismate is converted into prephenate through an intramolecular rearrangement via chorismate mutase (CM). Subsequently, prephenic acid can be decarboxylated by prephenate dehydratase (PDT) into phenylpyruvic acid (losing a hydroxyl group at the same time), which in turn undergoes a transamination via phenylpyruvate aminotransferase (PPY-AT) resulting in L-Phe. In the presence of a dehydrogenase, prephenic acid can undergo one more decarboxylative aromatization with retention of the hydroxyl group, leading to the formation of 4-hydroxylphenylpyruvic acid that would be an intermediate for tyrosine biosynthesis (not shown). From 4-hydroxylphenylpyruvic acid another transamination can occur, giving rise to Tyrosine (Tyr) and Tryptophan (Trp), further intermediates for the phenylpropanoid pathway. If prephenic acid undergoes transamination before a decarboxylative aromatization occurs, L-arogenic acid is formed catalyzed by prephenate aminotransferase (PPA-AT). Phe and Tyr can be synthetized as well from arogenic acid through arogenate dehydratase (ADT) and arogenate dehydrogenase (ADH, not shown), respectively [42,49].

The phenylpropanoid pathway generates the basic structure for the formation of flavonoids, hydroxycinnamic acids, coumarins, and stilbenes among other phenolics. This pathway starts with the deamination of L-Phe into cinnamic acid by the phenylalanine ammonia lyase (PAL) enzyme. P-coumaric acid is subsequently originated from the hydroxylation of cinnamic acid mediated by cinnamic acid-4-hydroxylase (C4H) [42,49]. Cinnamic acids can be synthetized as well from p-coumaric acid through hydroxylation and methylation reactions. Both cinnamic acid and p-coumaric acid can exist in plants in their free or esterified form. Hydroxybenzoic acids can be obtained by double bound cleavage of cinnamic acid and loss of two carbons from their side chain. The phenylpropanoid pathway ends with the conversion of p-coumaric acid in p-coumaroyl-Coenzyme-A (CoA) by the enzyme 4-coumarate:CoA ligase (4CL). P-coumaroyl-CoA will act as an intermediate in the biosynthesis of several other classes of phenolic compounds, such as flavonoids [42,49].

Flavonoid biosynthesis (Figure 4) starts with condensation of three molecules of malonyl-CoA with p-coumaroyl-CoA to form 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone) catalyzed by chalcone synthase (CHS) [52]. This is an iterative multistep reaction generating linear di-, tri-, and tetra-ketide-CoA intermediates as well as a cyclic and prochiral coumaryl-trione intermediate [52,53]. In addition, leguminous plants express the enzyme chalcone reductase (CHR) reacting to the intermediates produced by CHS forming 4,2′,4′-trihydroxychalcone (deoxychalcone) [52]. Deoxychalcone is the start point for the enzymatic conversion to 6′-deoxychalcone (isoliquiritigenin) catalyzed by CHS in addition to polyketide reductase (PKR) [42]. The resulting products of the two branches (naringenin chalcone and isoliquiritigenin) have the basic flavonoid structure C6-C3-C6. In the following step a ketoester will be formed by a Diekmann condensation (a specific Claisen cyclization) supported by chalcone isomerase (CHI) [49]. CHI enables the formation of the six atom O-heterocycle, which results in the formation of flavanones called naringenin if the educt was naringenin chalcone or liquiritigenin coming from isoliquiritigenin [42]. Different flavonol hydroxylases subsequently convert naringenin in dihydrokaempferol, dihydromyricetin and dihydroquercetin, which differ in their number of hydroxyl

groups. Flavanones are also precursor molecules to isoflavones, belonging to another subclass of flavonoids, the isoflavonoids (not shown in Figure 4) [49]. These compounds present a slightly different chemical structure in which ring B has migrated to the adjacent C3 carbon of the heterocycle in a process that is enzymatically catalyzed and quite rare in nature [49].
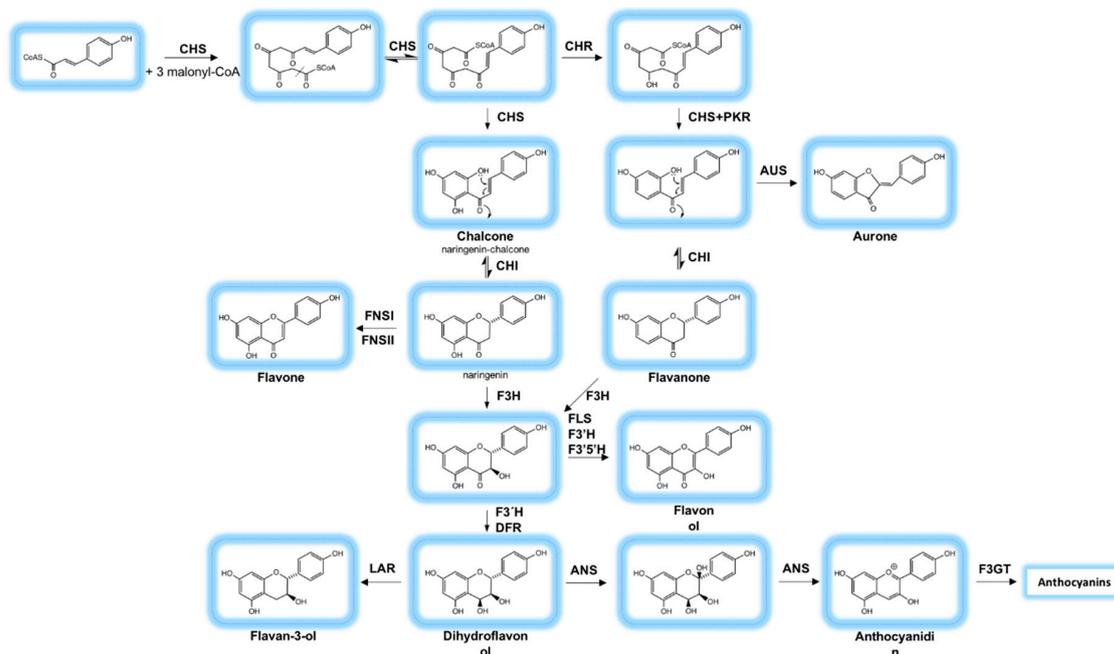
Figure 4. Biosynthesis of flavonoids via the flavonoid pathway. A detailed description can be found in the text. CHS: Chalcone synthase; CHR: Chalcone reductase; PKR: Polyketide reductase; CHI: Chalcone isomerase; AUS: Aurone synthase; FNSI: Flavone synthase I; FNSII: Flavone synthase II; F3H: Flavanone-3-hydroxylase; FLS: Flavonol synthase. *Anthocyanin pathway*: F3'H: Flavonoid-3'-hydroxylase; F3'5'H: flavonoid-3',5'-hydroxylase; DFR: Dihydroflavonol-4-reductase; LAR: leucoanthocyanidin reductase; ANS: Anthocyanidin synthase; F3GT: UDP-Glc flavonoid 3-O-glucosyltransferase (Adapted from references [49–51]).

Flavone synthase (FNS) converts flavanones into flavones, and through another pathway, flavanones are converted to flavanonols by the joint action of flavanone-3-hydroxylase (F3H), flavonoid-3'-hydroxylase (F3'H), flavonoid-3',5'-hydroxylase (F3'5'H) and flavonol synthase (FLS) [42]. Intermediates of these conversions, the dihydroflavonols, can give rise to flavandiols (leucoanthocyanidins) via dihydroflavonol-4-reductase (DFR) [42,49]. Flavandiols are the precursors of two further subclasses named flavan-3-ols and anthocyanidins. The formation of flavan-3-ols from flavandiols is mediated by the enzyme leucoanthocyanidin reductase (LAR) [42].

Anthocyanin biosynthesis starts when the dihydroflavonols are converted by DFR into the colorless anthocyanidin compounds leucocyanidin, leucopelargonidin and leucodelphinidin [42,49]. Removal of hydrogens and the induction of a double bond of ring C at C2 and C3 positions is mediated by anthocyanidin synthase (ANS) and the resulting ring C is a pyrilium kation [42]. From this reaction occurs the formation of the colored anthoyanidins, namely, cyanidin, pelargonidin, and delphinidin [49]. The addition of different chemical groups to anthocyanidins, e.g., methylation or coumaroylation, result in the stabilization of the compound and thereby a stabilization of the color as seen in fruits [42]. The enzyme UDP-glucose flavonoid 3-O-glucosyltransferase (F3GT) is responsible for converting the free anthocyanidin into its glycosylated form (anthocyanin) by the addition of a sugar unit [42,44,49]. The most common glycosylation site is the 3-hydroxyl position often using a glucose residue [43] (notice in Figure 2 the general nomenclature for the numbering of flavonoid carbons). However, if there is already a sugar unit present at the 3-hydroxyl position, the next position, were glycosylation

can occur, will be the 5-hydroxyl position [43]. Other sugars can be substitutes as well, e. g. arabinose, although glucose is the most common one. Also, anthocyanins can be acetylated, where an organic acid is esterified to the sugar [42]. The color of anthocyanidins varies with the pH, for example, these compounds are often red at a pH 3.5. As the pH increases they start becoming colorless and then shift towards blue [44,48]. Anthocyanins accumulate in the vacuole of the cell, and are often abundant in cells closer to the epidermis of the fruit [48]. Some enzymes can convert anthocyanidins into the correspondent flavan-3-ols in the biosynthesis of condensed tannins [42]. These resulting flavan-3-ols can polymerize to also form proanthocyanidins [54,55].

In plants, flavonoids may occur in the forms of O- or C-glycosides, with O-glycosides being the most usual structures [43]. Glycosylation can occur in positions 3-, 5- and 7- making the structures more water soluble, and allowing their storage in the vacuoles [42,45]. The most usual sugars involved in glycoside formation are glucose, galactose, rhamnose, xylose, arabinose and disaccharides such as rutose [43].

2.3. Genes and Regulation of the Biosynthesis of Phenolic Compounds in General

As described in the previous section, a great variety of enzyme-catalyzed reactions are involved in the phenolic and flavonoid biosynthesis. Nevertheless, only few of the involved genes in *Capsicum annuum* are known. Therefore, we will summarize here the actual knowledge of studies from other species mostly performed in Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), Petunia (*Petunia hybrid* (Sweet) D. Don ex W. H. Baxter), maize (*Zea mays* L.), and tomato (*Solanum lycopersicum* L.), and indicate those genes, that are already known.

Most of the involved enzymes known to date are thought to be located and to operate in the cell's cytosol [42,50]. In the various branches of the phenylpropanoid pathway, the most important enzymes include various transferases, reductases, membrane-bound cytochrome P450-dependent mono-oxygenases (P450s), and soluble non-heme dioxygenases. Figures 3 and 4 summarize the main biosynthetic enzymes involved in the shikimate, phenylalanine, and phenylpropanoid pathways.

Other enzymes are also involved in the hydroxylation, glycosylation and methylation of flavonoids. These structural modifications have as well an impact on the regulation of flavonoid biosynthesis, as alterations during biosynthesis can inhibit or turn the biosynthetic pathway in other directions [50].

Enzymes involved in these pathways are also believed to be organized in multi-enzymatic complexes associated with the endoplasmatic reticulum or anchored through P450 enzymes working in a synchronized, multistep way [50]. This would allow a channeling of the pathway in order to produce a specific flavonoid.

To better understand the regulation of the flavonoid biosynthesis pathway, various model species have been used as mentioned before to uncover genes involved in this complex pathway. Vermerris and Nicholson [42] briefly list a few examples of genes involved in the phenolic pathway, most of them identified in the aforementioned model species. Chalcone synthase (CHS) is encoded by *At5g13930* in Arabidopsis [56], *At3g55120* encodes for chalcone isomerase (CHI) [57], *At3g51240* for flavanone-3-hydroxylase (F3H) [58] and *At5g07990* for flavonoid-3'-hydroxylase (F3'H) [59].

In maize the CHS gene is called *COLORLESS1* (*C1*, *Zm00001d052673*) [60] as mutations in this gene result in loss of color, and F3'H was first described as *PR1* (*PURPLE/RED ALEURONE1*, *Zm00001d017077* [61]). Until today the analogous genes of CHI and F3H in maize are not described. In tomato some of the conserved genes were identified on the basis of gene ontology, like CHS, that is encoded at least twice, *CHS1* (*Solyc05g053550*) and *CHS2* (*Solyc09g091510*) [62], CHI, that exist as an analogue called *CHI1* (*Solyc05g010320*) [63] and a closely related gene called *CHIL* (*CHALCONE ISOMERASE-LIKE*, *Solyc05g052240*) [64], *F3H* (*Solyc02g083860*) [65], and *F3'H* (*Solyc03g115220*) [64]. For the last two only one member was yet identified each in tomato.

Genes involved in the biosynthesis of anthocyanins were also studied. For example, *At5g42800* encodes for DFR in Arabidopsis [66], in maize the *ANTHOCYANINLESS1* (*A1*, *GRMZM2G026930*) [67] gene encodes for this enzyme, and in tomato this is *Solyc02g085020* [68]. Formation of anthocyanidins

by the glycosylation of anthocyanins is also enzyme catalyzed and regulated. In maize, F3GT is encoded by the *BRONZE1* (*Bz1*, *Zm00001d045055*) [69] gene and in tomato by *Solyc10g083440* [63]. The enzyme dihydrokaempferol-4-reductase reduces leucoanthocyanidins back to 2,3-*trans*-flavonol, a reaction that is important in the formation of condensed tannins, although the gene encoding for this enzyme is still not fully elucidated. It is believed that *At1g61720* (*BANYULS*) in Arabidopsis encodes for an enzyme capable of using anthocyanidins as substrate to originating 2,3-*cis*-flavonols [70], a slightly different structure, while for the other species no analogues gene has been identified thus far. Nevertheless, the *trans*-flavonol is the basic structure for condensed tannins.

Transcriptional regulation is the most important tool for modulating flavonoid biosynthesis. Having a series of different branches inside the pathway coming from the same precursor but leading to different compounds makes this biosynthetic pathway very complex, requiring fine regulation [50]. Multiple genes are normally involved in such regulation along with different transcription factors (TFs). Therefore, the synthesis of flavonoids and other phenolic compounds can be regulated through a number of internal and external factors. The regulatory genes are involved in the expression of TFs belonging to the MYB-, basic helix-loop-helix (bHLH-), and/or WD-repeat protein families controlling flavonoid biosynthesis [71]. These TFs can enhance the expression of the structural genes involved in the pathway.

To date, the best studied part of regulation in flavonoid biosynthesis focuses on anthocyanins. Some of these genes were reviewed by [42]. Maize was the initial model species for analysis of the flavonoid biosynthesis pathway often based on maize seed color. For example, in maize, the genes *RED COLOR* (R) and *BOOSTER* (B) appear to be able to activate the F3GT-analogue of maize *Bz1*. Another gene, the *Lc (red leaf color1)* gene, encodes a TF required for the expression of *COLORLESS1* (*C1*) and *A1* (*DFR*) (both enzymes involved in the anthocyanidin pathway), while *A1* could as well be activated by B. In turn, C1 regulates the expression of *C2*, *A1*, *Bz1*, *Bz2* (*ZmGST*) and *A2* (*ZmANS*) genes [42].

At5g35550 (*TT2*) [72], *At4g09820* (*TT8*), *At5g24520* (*TTG1*) [73], and *At2g37260* (*TTG2*) [74,75] are some regulatory genes present in Arabidopsis. *At5g24520* (*TTG1*) encodes for a protein with four WD40 repeats, *At4g09820* (*TT8*) encodes for a protein important in the expression of *DFR* and *BANYULS*, *At5g35550* encodes for a R2R3 MYB protein that works together with the protein expressed by *At4g09820* (*TT8*) to regulate the expression of *BANYULS*. *TTG2* encodes a TF characterized by the presence of a WRKYGQK amino sequence resembling a zinc finger motive. This TF can be called the WRKY factor and it is believed to be involved in the synthesis of condensed tannins [76].

Concerning the regulation of the biosynthetic genes in *Capsicum* species, some studies have been conducted. However, most of them are focused on anthocyanin biosynthesis regulation and use purple pepper varieties, whereas anthocyanin accumulation can be more easily seen.

Aguilar-Barragán et al. [77] studied the effects of different TFs (MYB and WD40) on the anthocyanin biosynthetic pathway in chili pepper fruits (*Capsicum eximium*). The silencing of both TFs decreased the anthocyanin accumulation in chili peppers as the expression of the genes encoding for *CHS*, *CHI*, *F3′5′H*, *DFR*, and *F3GT* was down regulated [77]. Two different cultivars of *C. annuum* L. fruits, "Árbol" and "Uvilla", were used to elucidate anthocyanin accumulation patterns [78]. The expression of *F3′5′H*, *DFR*, *F3GT*, and *GLUTATHION S-TRANSFERASE* (*GST*, probably involved in the sequestration of anthocyanins) showed a positive correlation with anthocyanin accumulation.

First interactions between MYB and MYC TF in anthocyanin accumulation were uncovered by Stommel et al. [79]. Both *MYC* transcripts and MYC protein levels were higher in *Capsicum* tissues that presented high anthocyanin contents. The transcription of the genes encoding for MYC and MYB was also substantially higher in pigmented fruits and flowers reinforcing that these TFs are strongly correlated with the accumulation or depletion of anthocyanins. However, this positive correlation could not be found in other types of tissues [79].

In a study from 2011, Li et al. [80] hypothesized that three R2R3-MYB homologs might be involved in anthocyanin accumulation in leaves, fruits and flowers of green hot pepper (cultivar "Haijiao"). Only CaMYB1 (GenBank: AF477956.1) and CaMYB2 (GenBank: AJ608992) clustered with MYB

genes from other species in a phylogenetic analysis, while CaMYB3 (GenBank: EF222025) did not. Therefore, CaMYB3 was suggested to be a minor actor in the biosynthesis pathway. The expression of CaMYB1 and anthocyanin biosynthesis genes *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *F3GT* decreased with fruit ripening, suggesting that CaMYB1 is in fact regulating anthocyanin synthesis during fruit maturation. The authors also reported that the expression of *CaMYB3* decreased only slightly during fruit ripening. *CaMYB2* showed high expression in flowers and a dramatically reduced expression in ripening fruits, while *CaMYB1* showed only a weak expression in flowers. This is giving a hint, that both, CaMYB1 and CaMYB2 might regulate structural genes, as all analyzed structural genes of the flavonoid and anthocyanidin biosynthesis also showed a high expression in flowers of pepper [80].

In another study, the CaMYB2 TF was silenced by virus induced gene silencing in purple peppers [81]. This resulted in a repressed expression of the genes *CHS, CHI, F3H, F3'5'H, DFR, ANS, F3GT, ANTHOCYANIN PERMEASE* (*ANP*), and *GST* and a decreased anthocyanin accumulation in the leaves. Moreover, leaves were also more susceptible to *Phytophthora capsici* infection suggesting that CaMYB2 might also be involved in defense against pathogens. It was also demonstrated that CaMYB2 is involved in the activation of a complex built by an MYB TF, a basic helix-loop-helix (bHLH) MYC protein and WD40 protein (together MBW complex) [79]. The MBW complex is described to interact with the promoters of late structural genes and marginally with that of early structural genes [72] in the anthocyanidin biosynthesis. In addition, CaMYB2 also positively regulated MYC, so that the expression of the MYC locus in the silenced CaMYB2 environment was reduced [81]. However, in this study, the WD40 TF was negatively regulated by CaMYB2 [81], although the opposite was reported before [79]. Therefore, the results indicate that the regulatory network of MYB within the anthocyanin biosynthesis of different Capsicum species may vary [81].

Although the *Capsicum annuum* genome was finally published in 2014, the chromosomal location of flavonoid synthesis genes and associated regulatory genes is still not fully elucidated [82,83]. However in 2013, Wahyuni et al. [84] had already investigated flavonoid quantitative trait loci (QTLs) and proposed chromosomal locations for flavonoid synthesis related genes. For instance, the *Ca-MYB12* gene is located at chromosome 1 and a new QTL related to increased synthesis of naringenin chalcone was detected at the same region. Also, on chromosome 1 expression QTLs (eQTL) for *CHS-1*, *CHS-2*, *CHI-2* and *FLS* were mapped. On chromosome 9, a metabolite QTL (mQTL) for naringenin chalcone was mapped, suggesting either that flavonoid regulation is a complex phenomenon involving different parts of the genome as eQTLs for *CHS-1*, *CHS-2*, *CHI-1*, *CHI-2* and *FLS-2* were also mapped on chromosome 9. An eQTL for *FLS-2* was mapped at 162.1 cM on chromosome 6 and was strongly associated with mQTLs for four flavone C-glycosides. Further, on chromosome 6 the *CHI-4* locus at 176.8 cM presented an eQTL for the *CHI-4* gene, and this locus is associated with the expression of *FLS-2* gene and with the flavone C-glycosides. This study showed that flavonoid QTL hotspots exist in the *Capsicum* genome and that the biosynthesis of these compounds is complex and involves a wide number of genes [84].

To bring more insights in the biosynthesis of flavonoids and its regulation in *C. annuum*, further investigations have to be performed.

3. Phenolic Composition of *Capsicum Annuum*

3.1. Preparation and Measurement of Phenolic Compounds from Plants

The accumulation of SM increases a crop's value as they can have a vast amount of applications, as mentioned before. Therefore, it is important to understand the chemical composition of *Capsicum annuum* in order to understand its bio-active potential, health-promoting effects as well as for increasing its value and applicability.

Phenolic and flavonoid contents are usually measured in plant extracts using methanol in varying concentrations as a solvent [85,86], but other solvents can be used as well, such as ethanol, n-hexane, chloroform, acetone and many more [87]. The extraction can last from a few minutes to hours, will be

performed with or without the help of ultrasonification, at different temperatures ranging from room temperature over 65 °C up to boiling [88], and with or without an additional solid phase extraction step [89]. This results in almost incomparable data, as solvation of phenolic compounds will vary according to the used extraction method. In addition to the inconsistent preparation of the samples, in the following presentation data can be expressed either in dry weight (dw) or fresh weight (fw). Results expressed in dw are more precise since large weight fluctuations (e.g., between tissues) caused by differences in water content do not occur. This is particularly important when comparing different tissues like leaves and fruits that have a very different water content which contributes to the tissues "total weight". The heterogeneity of results reported and discussed in chapter 3.2 can partially be explained by this fact.

For the colorimetric determination of the total phenolic content (TPC), three different methods are widely used. The Folin-Ciocalteu reagent is a common reagent for determination of TPC [90]. The reagent produces a detectable colored complex when reacting with phenolic compounds, based on the transfer of an electron from an antioxidant substrate to the reagent complex in (often) alkaline medium. The color complex is measured at a wave length of 660 nm, and quantification is based on comparison with a standard solution of a determined concentration of e.g., gallic acid resulting in gallic acid equivalents (GAE), or catechin resulting in catechin equivalents (CE). Nevertheless, the Folin-Ciocalteu reagent can interact as well with any other reducing substance. As most antioxidants present in plants are phenolic compounds, the intensity of the coloring can serve as an approximation for the total phenolic content. Besides the varying extract preparation, the initial concentration of the extract and the ratio with the reagent, as well as the incubation time will influence the results and make a comparison of results from different studies difficult to interpret.

An alternative is the antiradical activity assay based on the reduction of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which has a deep violet color with an absorption maximum at 520 nm [91,92]. Neutralization of the radical by an antioxidant will change the color to colorless or pale yellow. This allows an optical monitoring, and results are sometimes shown in % inhibition/DPPH reduction after a given time [93], antioxidant concentration required to reduce 50% of the DPPH present (EC_{50}) [94], IC_{50} (half maximal inhibitory concentration), antioxidant efficiency (AE = 1/EC50 × TEC50) (where TEC50 is the time needed to reach the steady state at EC50 concentration) [95], or expression of antioxidant activity as µmol equivalents of standards such as vitamin C [96] or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) [97]. All of these presentations do not care about the reaction kinetic. In addition, many aspects during the implementation can affect the results, such as absolute and relative concentrations of DPPH and sample [98], solvent polarity and hydrogen bonding strength [99], pH [100], temperature [101], and oxygen. Therefore, a careful setup and internationally standardized protocols should be used, otherwise results of different origin cannot be compared [102].

In addition, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) is commonly used in food chemistry to measure the antioxidant activity of food products, as it reacts in the presence of phenolic compounds, thiols and vitamin C. ABTS is a stable radical that presents a blue color absorbing at 734 nm when in its radical cation form (ABTS+) and is colorless in the neutral form [103]. Hydroxyl groups of phenolic compounds and thiols act as hydrogen donors, converting the blue-greenish ABTS+ cation to its neutral form and thereby causing a decrease in the absorption at 734 nm. The antioxidant potential of the various antioxidants tested is compared to that of Trolox given as Trolox equivalent antioxidative capacity (TEAC) [103–105]. It is noteworthy that ABTS and DPPH radicals have different antioxidant potentials and reaction kinetics when reacting with phenolic compounds. During the reaction, the ABTS radical has to be formed initially, while DPPH is already a stabilized radical by itself. Furthermore, both have different molecular sizes and can have different affinities for other compounds that are present in the samples, like carbohydrates, lipids, proteins etc. Therefore, DPPH and ABTS assays follow different types of chemical reactions giving different and sometimes opposite results.

Total flavonoid content (TFC) can be estimated with a colorimetric assay using aluminum chloride [106]. Briefly, aluminum chloride is capable of binding the 3- and 5-hydroxyl and 4-keto groups of flavonoids, forming a complex. The formation of this complex can be monitored by spectroscopy (UV and visible). The results are normally expressed as "a certain flavonoid" equivalents as different flavonoids can be used for calibration curves (luteolin, quercetin, naringin etc.). However, this method is relatively specific for flavonols and flavones.

Some authors like Kim et al. [107], Marin et al. [108] and Howard et al. [109] used liquid chromatography to determine the total flavonoid content, based on the addition of the different (few) flavonoid groups identified. This can be a very imprecise estimation of the true flavonoid amount since the identification and quantification is made by comparison with two or three commercially available flavonoid standards (usually aglycones) which do not fully represent the total amount of flavonoids that might exist in pepper.

3.2. Total Phenolic and Flavonoid Content in Capsicum

The general phenolic content and antioxidant properties of *C. annuum* fruits, leaves and other plant parts are given in Table 1. It is revealed that fruits have been much better studied than any parts of the plant in terms of phenolic metabolites. Further, only one chalcone was reported to date in pepper fruits: phloretin dihexoside, a dihydrochalcone and possibly a diglucoside of phloretin [110].

Table 1. Global antioxidant activity and phenolic content of *Capsicum annuum* L. in fruits, leaves and other tissues.

Parameter	Content				Content Expressed In	Reference
	Fruit (Pericarp)	Leaves	Stems	Other		
Phenolic Content						
Total Phenolic Content	6.97–12.19	-	-	-	mg CE/g f.w	[108]
	6.92–7.32	17.14	-	-	mg CE/g dw	[107]
	1.03 *	-	-	-	mg GAE/g fw	[111]
	52.27	-	71.34	64.07 (placenta)	mg GAE/g dw	[112]
Total Flavonoid Content	0.017–0.2024	-	-	-	mg/g fw	[108,109]
	0.00366–0.0054	0.0206	-	-	mg/g dw	[107]
	46.71	-	69.84	58.96 (placenta)	mg NAE/g dw	[112]
	0.3864	-	-	-	mg QE/g fw	[111]
Total O-glycosyl-flavones	1.00–9.43	-	-	-	mg/100g fw	[108]
Total C-glycosyl-flavones	0.84–2.45	-	-	-	mg/100g fw	[108]
Total hydroxyl-cinnamics	0.33–1.99	-	-	-	mg/100g fw	[108]
Antioxidant Activity						
Radical scaveng-ing activity (RSA)	17.32–48.95	-	-	-	TEAC/kg dw	[113]
DPPH	15.40–286.23	463.23	-	-	IC$_{50}$ μg 80% EtOH extract (v/v)/mL	[107]
	-	49.2–62.2	-	-	IC$_{50}$ μg/mL	[114]
	60.41 *	-	-	-	% of inhibition	[111]
ABTS	55.23–118.00	68.67	-	-	IC$_{50}$ μg 80% EtOH extract(v/v)/mL	[107]
	-	26.2–33.7	-	-	IC$_{50}$ μg/mL	[114]
	34.44 *	-	-	-	μM TEAC/g fw	[111]
β-carotene bleaching	42.75–66.98	-	-	-	% of inhibition	[109]

* Results only for the bell cultivar of *Capsicum annuum* L.; CE—catechin equivalents; GAE—gallic acid equivalents; fw—fresh weight; dw—dry weight; NAE—naringin equivalents; QE—quercetin equivalents; TEAC—trolox equivalent antioxidative capacity; IC50—half maximal inhibitory concentration.

Stems and other parts of the plant (like placenta) have not been thoroughly studied, although leaves obtained some more attention. Nevertheless, the knowledge of the TPC and TFC in these pepper tissues will increase our understanding of the secondary metabolism in Capsicum.

TPC of *C. annuum* fruits ranges between 6.97–12.19 mg CE/g fw [108] or is around 1.03 mg GAE/g fw [111], depending on the assay used to determine it. Mudrić and collaborators [113] compared

the radical scavenging activities of different *Capsicum* cultivars harvested at different sites and years using the DPPH method and obtained variations between 17.32–48.95 mmol TEAC/kg dw, confirming that cultivar and harvest year have an influence on the chemical properties of *Capsicum annuum* [113]. In the same study the TPC also ranged from 7.03–14.92 g GAE/kg dw, variance occurring once more between two different cultivars. The authors also reported differences in TPC and radical scavenging activities between the same cultivar harvested in different years (data not shown). According to Kim et al. [107], *Capsicum* leaves had a TPC of 17.14 mg CE/g dw, whereas the fruits ranged from 6.92–7.32 mg CE/g dw (difference between green and red fruits respectably), a value about 3 times lower than in the leaves. Chen et al. [112] reported a slightly higher amount of TPC in the stems than in the fruits, 71.34 versus 52.27 mg GAE/g dw and similar results were obtained for total flavonoid content. In anti-inflammatory tests performed with mice, Chen et al. [112] also reported that the solvent of the extract influences as well the therapeutic effect, from almost useless to effective, although not comparable with dexamethasone treatment. In addition, Kim et al. [107] reported that the leaves ethanolic extract has a higher antioxidant activity than extracts from fruits, in terms of DPPH inhibition activity, although the same was not seen in the ABTS assay.

Besides, flavonoids do not represent a high percentage of *Capsicum* weight. In a study of Marín et al. [108], the highest value reported is 0.02% of the fruit's fw. In the same study, the total flavonoids, total O-glycosylflavones and total C-glycosylflavones were assessed in four fruit maturity states (from immature green, to green, immature red and red) in the variety "Vergasa". The authors reported that these values varied according to maturity stage, being higher when the fruit was in the immature state and decreased as the fruit went from green to red ripe state, suggesting that a change in the fruit's flavonoid composition occurs during fruit ripening [108]. Howard et al. [109] reported that the total flavonoid content varies not only with the maturity stage, but also with the cultivar (for example, one of the cultivars studied, "Inferno", presented a high flavonoid content in both immature and mature states). The authors reported the same effect in the amount of total hydroxycinnamics, which seemed to decrease with fruit development.

Kim et al. [114] focused only on the antioxidant potential and phenolic compounds of the leaves of three different cultivars of *Capsicum annuum*: "Blackcuban", "Hongjinju", and "Yeokgang-hongjanggun". The antioxidant activity measured either through the ABTS assay or the DPPH method revealed that the values could vary significantly depending on the cultivar. In the same study the absolute amount of nine phenolic compounds was assessed and also showed variations between the cultivars, at least for some of the compounds [114].

Medina et al. [111] evaluated the antioxidant activity of pepper from different cultivars using both the ABTS and the DPPH method. The values obtained for bell pepper (sweet varieties of *Capsicum annuum*) were 34.44 µM of TEAC/g fw for the ABTS assay and 60.41% of inhibition for the DDPH assay. In both assays, the results varied significantly according to the cultivar of pepper tested, for instance, "Caribe" and "Bell" were the cultivars that showed highest antioxidant activity in both assays. The antioxidant activity of fresh pepper fruits was assessed by Howard et al. [109] based on their peroxyl radical quenching properties and expressed in % of inhibition of β-carotene bleaching. Once more the results varied depending on the cultivar and maturity stage.

All these findings suggest that *C. annuum* has a different chemical profile and bio-active potential in leaves and fruits according to the cultivar, leading to significant variations within the species. The harvest year and ripening stage affect the chemical composition of the fruits as well. In addition, it should be noticed that the overall concentration of antioxidative agents and phenolic content did not vary a lot between fruits and leaves. It is also possible to conclude that other parts of the plant, for instance stems, have a high quantity of bio-active compounds as well.

3.3. Quantification of Flavonoids in Capsicum

When it comes to identification and quantifying phenolic compounds in various parts of the *Capsicum* plant, once more the main focus has been given to the fruits. Table 2 shows the

different phenolic compounds that were identified and quantified in *Capsicum annuum* using liquid chromatography coupled to a mass spectrometry (LC-MS). Fruits are much more well studied, although in the cases that different parts of the plant were used for the quantification it is possible to see that other organs have considerable amounts of phenolics [107,112,115].

Table 2. Quantification of phenolic compounds from *Capsicum annuum* L. (a—content expressed in mg/kg fw; b—content expressed in mg/kg dw; fresh weight (fw); dry weight (dw) ID—Identified but not quantified).

	Identified In				Reference
	Fruit	Leaves	Stems	Other	
Class of Phenolic Compounds	a	a	a	a	
Phenolic acids					
Quinic Acid	ID	-	-	-	[116]
Hydroxybenzoic acids					
Gallic acid	2.53–1013.0	-	-	-	[111,113,116,117]
1-O-galloyl-β-D-glucose	ID	-	-	-	[116]
Protocatechuic acid	0.29–0.83	-	-	-	[113]
p-Hydroxybenxoic acid	420 [b]	-	-	260 [b] (placenta)	[112]
	2.88–6.27	-	-	-	[113]
p-Hydroxyphenylacetic acid	0.41–1.77	-	-	-	[113]
p-Hydroxybenzoic acid β-glucoside	ID	-	-	-	[110]
Vanillic acid	-	-	130 [b]	1020 [b] (placenta)	[112]
	3.07–13.29	-	-	-	[113]
Vanillic acid glucoside	4.02	-	-	-	[118]
Vanillic-acid-1-O-β-D-gluco-pyranosylester	ID	-	-	-	[110,116]
Hydroxycinnamic acids					
Caffeic acid	1320 [b]	-	-	120 [b] (placenta)	[112]
	0.55–13.7	-	-	-	[108,111,113]
Hydrocaffeic acid	3.03	-	-	-	[118]
Caffeoyl glucoside	2.59	-	-	-	[118]
5-O-Caffeoylquinic acid	0.36–23.33	260–360	-	-	[113,114]
5-O-Caffeoylquinic acid methyl ester	-	10–340	-	-	[114]
5-O-Caffeoylquinic acid butyl ester	-	20	-	-	[114]
Caffeic acid-4-O-β-D-glucopyranoside	ID	-	-	-	[110]
1-O-Caffeoyl-β-D-glucopyranoside	ID	-	-	-	[110]
Caffeoyl-O-hexoside	ID	-	-	-	[119]
2-Caffeoyl-L-tartaric acid	ID	-	-	-	[116]
4-O-caffeoylquinic acid	ID	-	-	-	[116]
Chlorogenic acid	1560 [b]	-	3820 [b]	230 [b] (placenta)	[112]
	2–17.9	-	-	-	[111,117]
p-Coumaric acid	4560 [b]	-	2980 [b]	820 [b] (placenta)	[112]
	0.09–6.97	-	-	-	[113,118]
Coumaroyl quinic acid	ID	-	-	-	[116]
p-Coumaryl tyrosine	6.81	-	-	-	[118]
p-Coumaryl glycolic acid	6.47	-	-	-	[118]
trans-p-coumaroyl-α-D-gluco-pyranoside	0.5–1.0	-	-	-	[108]
Ferulic acid	120 [b]	-	-	-	[112]
	2.18–12.45	-	-	-	[113]
Feruloyl-O-glucoside	2.5	-	-	-	[119]
Feruloyl hexoside	32–53.4	-	-	-	[120]
Feruloyl-β-D-glucose	ID	-	-	-	[116]
trans-feruloyl-α-D-gluco-pyranoside	0.9–1.0	-	-	-	[108]
trans-p-feruloyl-β-D-gluco-pyranoside	84–359 [b]	-	-	-	[121,122]
Syringic acid	0.66–1.21	-	-	-	[113]
Sinapic acid	0.45–1.89	-	-	-	[113]
Sinapoyl hexoside	65.0–72.4	-	-	-	[120]
trans-sinapoyl-α-D-gluco-pyranoside	1.4–3.0	-	-	-	[108]
trans-p-sinapoyl-β-D-gluco-pyranoside	74–419 [b]	-	-	-	[121,122]
Cinnamic acid	0.03–0.24	-	-	-	[113]
Coumarins					
Hydroxycoumarin	2.42	-	-	-	[118]
Daphnetine *(7,8-Dihydroxycoumarin)*	16.29	-	-	-	[118]
Aesculin *(Esculetin-6-β-D-glucoside)*	0.16–0.20	-	-	-	[113]
Umbelliferone *(7-hydroxycoumarin)*	10.66–14.61	-	-	-	[113]

Table 2. *Cont.*

	Fruit	Leaves	Stems	Other	Reference
Class of Phenolic Compounds	[a]	[a]	[a]	[a]	
Chalcones					
Phloretin dihexoside	ID	-	-	-	[110]
Flavonoids					
Flavonols					
Quercetin	8860 [b]	5.72 [b]	6510 [b]	10270 [b] (placenta)	[112,116]
	3.29–783.83	-	-	-	[107,109,113,117,118,123]
Quercetin-3-O-hexoside	13.5	-	-	-	[120]
Quercetin rhamnoside	82.60	-	-	-	[118]
Quercetin dihexoside	ID	-	-	-	[110]
Quercitrin (*Quercetin-3-O-rhamnoside*)	0.5–1501.8	-	-	-	[108,117,119,120]
Quercetin-O-rhamnosyl-O-hexoside	17.6–23.1	-	-	-	[120]
Quercetin glucoside	19.86	-	-	-	[118]
Quercetin-3-vicianoside	ID	-	-	-	[110]
Quercetin rhamnoside-glucoside	ID	-	-	-	[84]
Quercetin-3-O-glucoside-7-O-rhamnoside	ID	-	-	-	[84]
Quercetin-3,7-di-O-α-L-rhamno-pyranoside	ID	-	-	-	[110]
Quercetin-3,7-diglucuronide	ID	-	-	-	[116]
Quercetin-3-rhamnopyranoside	ID	-	-	-	[110]
Quercetin-3′-O-β-D-gluco-pyranoside	ID	-	-	-	[110]
Quercetin-4′-β-D-glucopyranoside	ID	-	-	-	[110]
Quercetin-3-O-α-L-rhamno-pyranoside	113–993 [b]	-	-	-	[121,122]
Quercetin-3-O-α-L-rhamnopyranoside-7-O-β-D-glucopyranoside	ID	-	-	-	[121]
Quercetin-3-O-(6′-O-acetyl)-β-D-glucopyranoside	ID	-	-	-	[116]
Hyperoside (*Quercetin-3-O-galactoside*)	1.81–27.28	-	-	-	[113]
Isoquercitrin	31.9–52.5	-	-	-	[117]
Rutin	1.05–52.0	-	-	-	[111,113,117]
Rutin pentoside	ID	-	-	-	[110]
Kaempferol	50 [b]	-	160 [b]	-	[112]
	128.0–143.0	-	-	-	[117]
Kaempferol-3-O-hexoside	ID	-	-	-	[124]
Kaempferol-di-glucoside	17.17	-	-	-	[118]
Kaempferol-feruloyl dihexoside	ID	-	-	-	[124]
Kaempferol-pentosyldihexoside	42.1	-	-	-	[120]
Kaempferol-O-pentosyldihexoside	1.3	-	-	-	[119]
Kaempferol-7,4′-dimethoxy-8-butyryl ester	ID	-	-	-	[116]
Myricetin	2280 [b]	-	-	1380 [b] (placenta)	[112]
Flavan-3-ols					
Catechin	-	-	30 [b]	-	[112]
	1.1–36.8	-	-	-	[111]
Epicatechin	1–79.3	-	-	-	[111]
Flavones					
Dihydroxyflavone-O-hexoside	3.1	-	-	-	[119]
Chrysoeriol-6,8-di-C-hexoside	ID	-	-	-	[124]
Apigenin	1.38	-	-	-	[113]
Apigenin-7-O-glucopyranoside	ID	-	-	-	[124]
Apigenin-C-pentosyl-C-hexoside	5.5–7.4	-	-	-	[120]
Apigenin-6,8-di-C-hexoside	ID	-	-	-	[84]
Apigenin-6-C-pentoside-8-C-hexoside	ID	-	-	-	[84]
Vicenin 2 (*Apigenin-6,8-di-C-glucoside*)	12.7	-	-	-	[120]
Apiin (*Apigenin-7-O-apioglucoside*)	0.27–0.43	-	-	-	[113]
Apigenin-7-O-β-D-apiofuranosyl (1→2)-β-D-glucopyranoside	ID	6–3530	-	-	[110,114]
Apigenin-8-C-glucoside	ID	-	-	-	[116]
Apigenin-8-C-α-L-arabinoside-6-C-β-D-glucoside	ID	-	-	-	[110]
Schaftoside (*Apigenin-6-C-β-D-gluco-pyranoside-8-C-α-L-arabinopyranosid*)	18–109 [b]	-	-	-	[121,122]
Diosmetin-7-O-β-D-glucoside	ID	-	-	-	[110]
Vitexin	0.15–3.50	-	-	-	[113]

Table 2. *Cont.*

	Identified In				Reference
	Fruit	Leaves	Stems	Other	
Class of Phenolic Compounds	[a]	[a]	[a]	[a]	
Luteolin	260 [b]	14.34 [b]	280 [b]	310[b] (placenta)	[112]
	0.16–103.50	-	-	-	[107,109,111,113,118,123]
Luteolin-glucoside	5.09	-	-	-	[118]
Luteolin-di-glucoside	5.66	-	-	-	[118]
Luteolin-C-pentosyl-C-hexoside	8.0–17.4	-	-	-	[120]
Luteolin-6-C-hexoside	7.7	-	-	-	[120]
Luteolin-8-C-hexoside	19–26.5	-	-	-	[120,124]
Orientin *(Luteolin-8-C-glucoside)*	12.7	-	-	-	[120]
Isoscoparin *(Chrysoeriol-6-C-glucoside)*	23.4	-	-	-	[120]
Luteolin-6-C-pentoside-8-C-hexoside	ID	-	-	-	[84]
Luteolin-6,8-di-C-hexoside	1.7–12.9	-	-	-	[120]
Luteolin-C-6-malonyl-C-pentoside	ID	-	-	-	[124]
Luteolin-6-C-β-D-glucopyranoside-8-C-α-L-arabinopyranoside	59–299 [b]	-	-	-	[121,122]
Luteolin-O-(apiosylacetyl)-glucoside	6.1-19.2	-	-	-	[120]
Luteolin-O-maloylpentosyldi-hexoside	105.3	-	-	-	[120]
Luteolin-O-(apiosylmalonyl)-glucoside	60.1–206.6	-	-	-	[120]
Graveobioside A *(Luteolin-7-O-[2-(α-D-apiofuranosyl)-α-D-glucopyranoside])*	160–1367 [b]	-	-	-	[121,122]
Graveobioside A *(Luteolin-7-O-β-D-apiofuranosyl-(1->2)-β-D-glucopyranoside)*	4.179	4.1 –121.36	-	-	[114,115,121]
Graveobioside A *(Luteolin-7-O-apiosyl-glucoside)*	ID	-	-	-	[124]
Graveobioside A *(Luteolin-7-O-(2-apiosyl)glucoside)*	0.1	-	-	-	[119]
Luteolin-7-O-(2-apiosyl-6-malonyl)glucoside	3.9–41.4	-	-	-	[108]
Luteolin-7-O-[2-(β-D-apiofurano-syl)-4-(β-D-glucopyranosyl)-6-malonyl]-β-D-glucopyranoside	47-412 [b]	-	-	-	[121,122]
Cynaroside *(Luteolin-7-O-glucopyranoside)*	1.00–7.21	30-370	-	-	[113,114]
Isoflavones					
Genistein-4,7'-dimethyl ether	ID	-	-	-	[116]
6'-O-acetyl-Daidzin	ID	-	-	-	[116]
Flavanones					
Naringenin	1.12–4.46	-	-	-	[113]
Naringenin-chalcone-hexose	ID	-	-	-	[110]
Naringenin-7-O-β-D-glucoside	ID	-	-	-	[110]
Narigenin-7-O-β-D-(3''-p-couma-royl)-glucopyranoside	ID	-	-	-	[110]
Theaflavanoside I	ID	-	-	-	[110]
Flavanonols					
7-O-β-D-Glucopyranosyl-dihydroquercetin-3-O-α-D-glucopyranoside	ID	-	-	-	[110]
Anthocyanidins					
Nasunin *(Delphinidin-3-(p-coumaroylrutino-side)-5-glucoside)*	14.7–286.2	1525	-	-	[125,126]
Delphinidin-3-[4-(*trans*-coumaroyl)-L-rhamnosyl (1→6)glucopyrano-side]-5-O-glucopyranoside	-	860	-	-	[114]
Myrtillin *(Delphinidin-3-O-gluco-side)*	2.2	-	-	-	[125]
Tulipanin *(Delphinidin-3-O-ru-tinoside)*	2.9	-	-	-	[125]
Delphinidin-3-rutinoside-5-glucoside	1.5	-	-	-	[125]
Delphinidin-3-rhamnoside	2.4	-	-	-	[125]
Delphinidin-3-caffeoylrutinoside-5-glucoside	7.8	-	-	-	[125]
Stilbenes					
Resveratrol	3.8–12.2	-	-	-	[111]
Other phenolics					
Vanillin	0.19–0.85	-	-	-	[113]
Conyferyl aldehyde	0.26–0.43	-	-	-	[113]
2'-O-β-Apiofuranosyl-arbutin	ID	-	-	-	[110]
Lariciresinol-glucopyranoside	ID	-	-	-	[110]

Kashiwagi et al. [115] and Kim et al. [114] showed that pepper leaves have a higher amount of quercetin, luteolin and graveobisode A than the fruits. In addition, for the cited studies different cultivars of *Capsicum annuum* L. have been used, which justifies the ranges reported for the compounds

quantified. Morates-Soto et al. [110] used the cultivars "Italian sweet" (green), "Lamuyo" (yellow) and "California wonder" (red). Mokhtar et al. [118] used the "Biskra" fruits variety and Park et al. [119] used the "Chelsea" fruit cultivar. Marín et al. [108] analyzed the "Vergasa" cultivar at four different fruit maturity states. Sadilova et al. [125] analyzed the anthocyanins present in a violet pepper variety. Mudric et al. [113] studied different cultivars and varieties of *Capsicum* ("Lemeška" sweet, "Lemeška" hot, "Lemeška" semi-hot and "Lakošnicka") harvested in different years. Ogunruku et al. [117] analyzed the variety "Grossum" in ripe and unripe maturity stages. Materska et al. [121] studied two hot pepper cultivars, "Cyklon"and "Bronowicka Ostra", and two semi hot cultivars, "Tajfun" and "Tornado", at two different maturity states. Tundis et al. [127] used the cultivars "Acuminatum" small and "Cerasiferum" and Moreno-Ramírez et al. [116] studied the chili variety "Glabriusculum". Jeong et al. [120] analyzed different genotypes from *Capsicum annuum* L. (CLCu, CLOg and CLST), whereas Jang et al. [124] used "CM334" but different harvest times were analyzed in this study. Wahyuni et al. [84] analyzed four different species of *Capsicum, Capsicum annuum, Capsicum chinense, Capsicum baccatum* and *Capsicum frutescens*.

Besides the above mentioned dependencies, *Capsicum annuum* phenolic composition depends as well on several other factors, such as the ripening stage [109], cultivar [110,127], harvesting location and year [113]. For instance, Materska et al. [121] studied fruits from four different cultivars at two different maturity stages, harvested in three different years. For the eight compounds quantified in this study, the concentrations varied according to the used cultivar and maturity level. The amount of graveobioside A ranged from 160 to 1367 mg/kg dw. The same was true in a study from Tundis et al. [127] on two different *Capsicum* cultivars for the amounts of quercetin, luteolin and kaempferol. The amount of quercetin and luteolin varied between green, yellow, orange and red pepper [128]. The highest concentration of quercetin was found in yellow and green pepper fruits (29.0-29.6 µg/g fw) while a higher amount of luteolin was reported in red pepper (11.0 µg/g fw).

So far phenolic acids and flavonoids were identified in fruits, leaves, stems and placenta of pepper. From the phenolic acids family, hydroxybenzoic acids and hydroxycinnamic acids were reported. Other families of phenolic compounds are also present, like chalcones, stilbenes and coumarins. Nevertheless, the flavonoid class has the highest quantity of SM identified in *Capsicum*, as compounds belonging to the flavonol, flavan-3-ol, flavone, flavanone, flavanonol, flavanol and anthocyanidin subfamilies are reported.

Hydroxybenzoic acids are phenolic acids which have a carboxyl group substituted on a phenol. Other types of phenolic acids are the hydroxycinnamic acids. They derive from cinnamic acid, have a phenyl propane backbone and can commonly be esterified with quinic acid [42]. Phenolic acids from both subfamilies are reported in *C. annuum* L. fruits, leaves, stems and placenta. The major hydroxybenzoic acids present in *Capsicum* fruits are gallic acid (maximum reported 1013.0 mg/kg fw) and vanillic acid (13.29 mg/kg fw) [111–113,117]. Vanillic acid is present in *Capsicum* stems and placenta, and p-hydroxybenzoic acid was also detected in pepper's placenta [112]. Various hydroxycinnamic acids have been reported in pepper, namely caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, syringic acid, sinapic acid and cinnamic as well as derivatives from the mentioned acids. Caffeic acid was identified in the placenta of *C. annuum* [112], 5-O-Caffeoylquinic acid is also present in the leaves [114] and chlorogenic and p-coumaric acids were identified in both stems and placenta [112]. Interestingly, 5-O-caffeoylquinic acid methyl ester and 5-O-caffeoylquinic acid butyl ester where only reported in leaves [114].

Coumarins are lactones of hydroxycinnamic acid that have a C6-C3 structure [42]. In *Capsicum* fruits, only four coumarins have been identified to date. Daphnetine and umbelliferone, two hydroxycoumarins, are present in high amounts of 16.29 and 14.61 mg/kg fw respectively [113,118].

Flavonols are perhaps one of the major flavonoid subfamilies present in peppers. They are structurally similar to flavones, with an additional hydroxyl group present on carbon 3 of ring C. Quercetin and its derivatives are the major flavonols present in *Capsicum*, with quercetin (783.83 mg/kg fw) [107,109,113,117,118,123] and quercitrin (1501.8 mg/kg fw) [108,117,118,120] occurring

in high contents. Other flavonols like kaempferol and its glycosides were also reported with 1.3–160 mg/kg fw. Once again most of the flavonols were identified only in fruits. Nevertheless, quercetin was also identified in leaves, stems and placenta of *C. annuum* [112]. In the case of this flavonol, placenta presented the highest content with 10,270 mg/kg dw. Kaempferol is also present in the stems of *Capsicum* in a higher quantity than in fruits. Finally, myricetin was as well present in the placenta of *C. annuum* (1380 mg/kg dw) [112].

Flavan-3-ols have a completely saturated ring C and a hydroxyl group at the third carbon. In *Capsicum*, only 2 flavan-3-ols have been reported, catechin, in both stems (30 mg/kg dw) and fruits (1.1–36.8 mg/kg fw) and epicatechin only in fruits (1–79.3 mg/kg fw) [111,112].

Flavones are also a major flavonoid subfamily present in pepper, and are characterized by having a ketone group and an unsaturated bond between the second and third carbon of the C ring. Luteolin-O-(apiosylmalonyl)glucoside (206.6 mg/kg fw) [120], luteolin-O-maloylpentosyldihexoside (105.3 mg/kg fw) [120] and luteolin in its aglycone form (103.5 mg/kg fw) [107,109,111,113,118,123] are the major flavones present in the fruits of *Capsicum*. In the leaves, the amount of graveobioside A is even higher than in the fruits (reaching 121.36 mg/kg fw) [114,115,121], cynaroside, another glycoside of luteolin also exist in considerable amounts in the leaves (370 mg/kg fw) [113,114]. However, the main flavone present in pepper leaves is apigenin-7-O-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside reaching up to 3530 mg/kg fw [109].

Only two **isoflavones** have been identified (but not quantified) to date in *Capsicum* fruits, namely genistein-4,7′-dimethyl ether and 6′-O-acetyl-daidzin [116].

Flavanones are structurally very similar to flavones, with the exception of an absent double bound between second and third carbon of ring C. Only four flavanones have been identified in *C. annuum* fruits, with all being derivatives of naringenin. Naringenin has been the only flavanone quantified, ranging from 1.12 to 4.46 mg/kg fw [113].

One **flavanonol** has been identified but not quantified in *Capsicum annuum*, 7-O-β-D-glucopyranosyl-dihydroquercetin-3-O-α-D-glucopyranoside, a diglucoside of taxifolin [110]. Structurally similar to flavanones, they are characterized by having a hydroxyl group linked to the third carbon of ring C.

Finally, **anthocyanidins** are characterized by having the ring C in the form of a pyrilium kation and are typically found as glycones (linked to a sugar moiety) forming structures known as anthocyanins. In *C. annuum* fruits the anthocyanin content is mainly constituted by delphinidin derivatives. The most abundant anthocyanin is nasunin (delphinidin-3-(p-coumaroylrutinoside)-5-glucoside), being present in purple pepper fruits (14.7–286.2 mg/kg fw) [125]. The content of this anthocyanin in the leaves of *C. annuum* is much higher (1525 mg/kg fw) [125,126]. Another anthocyanin, delphinidin-3-[4-(*trans*-coumaroyl)-L-rhamnosyl-(1→6)-glucopyranoside]-5-O-gluco pyranoside, was reported only in the leaves of *Capsicum*, in an amount of 860 mg/kg fw [114]; Ichiyanagi et al. [129] also reported this compound as an *trans*-isomer of nasunin.

Stilbenes belong to the phenolic compound family and share partially the same biosynthetic pathway as flavonoids. They have a C6-C2-C6 backbone and the general structure of two benzene rings linked together by an ethene bridge. Only one stilbene has been identified in *C. annuum* L. fruits, resveratrol, whose amount ranges from 3.8–12.2 mg/kg fw [111].

Other **phenolics compounds** that have also been reported but not further quantified in *Capsicum*, are 2′-O-β-apiofuranosyl-arbutin, a glycosylated hydroquinone, and lariciresinol-glucopyranoside a glycosylated lignin [110]. Vanillin and coniferyl aldehyde, two phenolic aldehydes, have also been quantified in the fruits, although in small amounts (0.19–0.85 and 0.26–0.43 mg/kg fw respectively) [113].

As described previously and illustrated in Table 2, most studies on the phenolic composition of pepper have had a strong focus on the fruits. To date, only Chen et al. [112] reported the chemical compositions in leaves, stems and placenta of *C. annuum*. The leaves alone have been studied only by Kim et al. [114], Kashiwagi et al. [115] and Lightbourn et al. [126], with the number of compounds identified being far less than the ones found in fruits [114,115,126]. Nevertheless, these studies give us

important information regarding the distribution of phenolic compounds in *C. annuum*, and how it can vary according to the analyzed tissue. For example, in the study of Chen et al. [112], one can see that vanillic acid was only identified in stems and placenta of *C. annuum* and not in fruits or leaves; luteolin was also present in higher amounts in stems and placenta; catechin was only identified in stems in this study, and chlorogenic acid, quercetin and kaempferol were present in higher concentration in stems comparing to the fruits. In the study of Kim et al. [114], a series of flavonoids were either only present in leaves (as it is the case of 5-O-caffeoylquinic-acid-methyl-ester, 5-O-caffeoylquinic-acid-butyl-ester, nasunin and apigenin-7-O-β-D-apio-furanosyl-(1→2)-β-D-glucopyranoside) or were present with higher quantities in leaves than in fruits (the case of 5-O-caffeoylquinic-acid, graveobioside A and cynaroside). Lightbourn et al. [126] also identified nasunin in pepper fruits, but once again concluded that the amount of this anthocyanidin was much higher in leaves.

These studies reflect that SM, namely flavonoids, are differently distributed in the pepper plant. Some of these metabolites exist in higher concentrations in other tissues of the crop than fruits. Further studies regarding the phenolic composition of different *Capsicum* plant tissues are needed in order to elucidate the distribution of SM in the plant.

4. Possible Strategies to Increase Phenolic Compounds in *Capsicum annuum*

There is compelling evidence that plant SM and especially flavonoids are beneficial for human health, which led to dietary recommendations for consumption of fruits and vegetables rich in bio-active compounds in recent years [1–3]. As these recommendation campaigns have been relatively unsuccessful so far, the idea of producing foods and food supplements with enhanced concentrations of SM to support human health and decrease micronutrient deficiency is worth considering, although no precise levels of SM concentrations can be recommended due to several factors [130]. Furthermore, increased levels of SM in residual biomass could be valuable for extraction purposes and usage for industrial applications.

Capsicum is a crop of major agricultural and economic importance and has been domesticated for several thousand years [131]. Early breeding approaches have focused on variety improvement regarding fruit size, shape and color, as well as non-pungency (bell-type peppers are commercially most important). Accordingly, many of the identified QTLs are related to these traits [132]. High-yield in combination with resistance to pests and diseases and protection against abiotic stress are strong selection criteria for breeding strategies. In addition, improvement of fruit quality for consumption (taste, smell, color and appearance to consumer) or usage in industry (processing, pungency) is also in the focus of breeders. Years of domestication of pepper, however, have led to loss of genetic diversity, leading to higher susceptibility to diseases and stress [82].

Several studies showed that peppers contain relevant amounts of beneficial phytochemicals, such as vitamins, capsaicinoids and phenolic compounds [40]. This in combination with the increasing consumption worldwide makes *Capsicum* an interesting target to improve the production of SM, especially flavonoids. As discussed above (chapter 3), the content and composition of phenolic compounds in fruits, and concomitantly the antioxidant activity, varies between different accessions. Therefore, a possible strategy to enhance the concentrations of beneficial SM could be the selection for cultivars with already high amounts of flavonoids for hybridization-based breeding [40]. In addition, wild *Capsicum* germplasm could be used for breeding, as it has been shown that non-domesticated species often contain higher levels of polyphenolics than cultivated varieties [133,134]. In general, broader studies looking into the flavonoid contents and phenolics composition of many different cultivated pepper accessions and wild species are necessary to provide a good foundation for targeted breeding approaches. Although generating a cultivar with the desirable trait (e.g., increased flavonoid content) without losing any other usual breeding criteria takes a long time, breeding programs have a large potential in generating cultivars with enhanced concentrations of phenolic compounds, as shown for cranberry, strawberry, peach and plum [135–137].

Our current knowledge about flavonoid pathways, their genes and regulation, as outlined above, provides the basis for metabolic engineering as a measure to increase flavonoid content, although further studies are necessary to pinpoint structural and regulatory genes of the respective biosynthesis pathways in pepper, as well as developmental and tissue-specific regulation.

Manipulation of secondary metabolism by genetic engineering has been proven successful in several plants, among others tomato, via several strategies: On the one hand, overexpression of structural genes of flavonoid pathways from different plant sources (e.g., *Petunia hybrida, Vitis vinifera*) in tomato was successfully used to overcome rate-limiting steps of the respective biosynthesis pathways leading to flavonoid accumulation or even producing new flavonoids [138–140]. In addition, downregulation of key genes of the lignin biosynthesis pathway in tomato (lignin shares common precursors with phenolic compounds) resulted in metabolic flux being redirected into the production of other phenolic compounds (e.g., chlorogenic acid, rutin) [141]. Another approach involves genetic manipulation of regulatory genes like TFs. Fruit-specific expression of maize TF genes in tomato led to an upregulation of the flavonoid pathway and high levels of kaempferol and naringenin were detected [142]. Similarly, two TFs from *Antirrhinum majus* caused accumulation of anthocyanin in tomato fruits, with concentrations being even comparable to those found in blackberries and blueberries [143]. The combination of several TFs expressed in tomato fruits did not only caused induction of the flavonoid biosynthesis pathway but even altered carbon flux and increased the supply from carbon metabolism [65].

The technical potential of metabolic engineering on pepper appears very promising, as shown by the few examples of successful manipulation of anthocyanin biosynthesis [77,81]. However, the commercialization of genetically modified pepper fruits, at least in Europe, might represent difficult. European consumers most likely will not accept genetically engineered plant products and even the relatively new, precise gene-editing technologies such as CRISPR-Cas9 have just been declared to fall under the same stringent regulations as do conventional genetically modified organisms.

A further approach to improve fruit quality of peppers, without time-consuming breeding or controversial genetic modifications, could be adjustment of environmental conditions during cultivation. It is known that plant SM are induced significantly in response to biotic or abiotic stress [144–146] and this has been reviewed in detail elsewhere [147]. Application of stress with the aim to improve product quality has mainly been investigated for medicinal plants (reviewed in reference [148]) but several studies about the effect of stress treatments on vegetables and fruits have also been performed [149–155]. Regarding phenolic compounds and flavonoids, an increase of up to 150% of lutein was found in broccoli under low temperature [150], whereas certain cultivars of strawberry would even increase anthocyanins up to 240% [151]. Light intensity during cultivation was also shown to affect polyphenols positively in apple [152] and tomato [153,154] even after harvest [155].

However, information regarding stress-effects on polyphenols in *Capsicum* is scarce. One study reported no or even negative effects of salt on phenolic compounds which were dependent on intensity of salinity and the maturity stage of the pepper fruit [156]. In contrast, positive effects of high salt concentrations on SM levels were observed in tomato and strawberry [157–159]. Furthermore, different cultivation conditions affected the accumulation of antioxidants in different sweet pepper cultivars [160]. Organic farming reduced the total phenolics content in both ripe and unripe fruits in twenty-five pepper accessions compared to conventional cultivation, whereas seven accessions showed induction effects under organic farming [161]. This shows that the choice of environment and pepper variety can enhance individual antioxidant potential of fruits to improve health benefits for consumers.

Elicitors are often used to encourage the production of SM such as flavonoids, terpenoids or alkaloids in plants [162,163]. Examples for potent elicitors include phytohormones such as jasmonate or salicylic acid (SA) and substances of microbial origin such as chitosan [164]. Jasmonates and in particular methyl jasmonate (MeJA) play a central role in biosynthesis of nearly all SM, e.g., flavonoids and alkaloids [165], and it has been reported that MeJA can induce production of phenolics and flavonoids in bell pepper and Arabidopsis [166,167]. In addition, foliar application of SA and chitosan

significantly increased the activity of PAL, the key enzyme of phenylpropanoid metabolism, which could suggest accumulation of phenolic compounds as a result [168].

The approach to selectively trigger the production of flavonoids or phenolic compounds in pepper fruits by controlled environmental stresses seems promising but requires further investigation. This includes more in-depth studies on *Capsicum* phenolic composition regarding the effect of different cultivation/stress conditions, stress intensity and duration. Also genotype x environment interactions need to be further investigated in order to identify suitable cultivar—stress/elicitor combinations with a large potential for induction of SM. A challenge will be to identify stress treatments that increase the levels of phenolic compounds in pepper fruits without causing any yield loss and are easily transferable and applicable in horticultural practices. A study in tomato showed that nitrogen and general mineral limitation applied in a commercial like greenhouse increased the concentrations of rutin and solanesol in leaves [169], however, stressed plants more frequently showed symptoms of blossom end rot on fruits.

Environmental stress will not only affect SM levels in pepper fruits but also affect the whole plant. Annual pepper cultivation produces considerable amounts of green biomass (1.3 kg m^{-2} per season) [170]. This biomass is currently underutilized as it is usually composted or used for combustion, although high amounts of SM and antioxidant activity has also been found, for example in pepper stalks [112]. Therefore, side streams of horticultural production could be used in combination with stress applications to extract SM for industrial purposes, which would enhance the resource efficiency of this food production system and will add an enormous value to this crop [171]. To circumvent any negative effects on fruit yield, stress treatments could be performed at the end of fruit production, targeting only the residual biomass. This bio-economic concept would represent an expansion of the current, horticultural value chain towards a cascade utilization of pepper biomass.

However, as outlined above, little is known about the composition of phenolic compounds in leaves and stems of peppers or their inducibility by stress, therefore these tissues need to be included in future experimental designs analyzing stress responses of peppers. A recent interdisciplinary study with tomato identified the key aspects for the valorization of tomato residual biomass by extraction of SM. These are the effective induction of SM content in leaves, but also the feasibility of stress applications in horticultural practice, economic factors (market value of SM versus additional cost for treatment, extraction, logistics) as well as socio-economic aspects like acceptance from the value-chain actor and consumer side ([171], and L. Junker personal communication).

The recent developments and studies investigating the secondary metabolism not only in fruits but also other plant tissues increased our understanding and showed the total potential of pepper plants. Nevertheless, our limited actual knowledge of the biosynthesis and distribution of phenolic compounds and in particular flavonoids in tissues other than fruits should be further investigated to develop targeted approaches for metabolic engineering, and/or tailoring cascades in horticulture. In addition, this implies the conceptualization of multi-disciplinary projects to cover the entire value-chain in horticulture, while also including socio-economic effects. As a first step a changed focus from anthocyanidins (as coloring agents) to other flavonoids would be preferable, as parts of these pathways are already well studied in pepper. In summary, peppers are a great resource of metabolites with protective impacts for the plant, and for coloring and flavoring our daily life.

Author Contributions: Conceptualization, A.W.; Writing-Original Draft Preparation, V.C.L., J.J.R., A.W.; Writing-Review & Editing, V.C.L., J.J.R., A.W.; Visualization, V.C.L., J.J.R., A.W.; Funding Acquisition, J.J.R., A.W.

Conflicts of Interest: The authors declare no conflict of interest

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