The influence of phosphate-availability and phytic acid on the profiles of fatty acids, (poly)phenols, carotenoids, and tocochromanols in maize (*Zea mays* L.) grains – from field experiments to human in vitro digestion studies

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This work is dedicated to my wife Astrid, my parents Barbara and Jürgen, my sisters Carina and Maria, and my grandparents Hildegard, Walter, Agnes, and Erwin.

“Ehrfurcht vor dem Leben” (“Reverence for Life”) – Albert Schweitzer

Summary

Phosphorus (P) is an essential element for living organisms and involved in phosphorylation reactions, including the biosynthesis of several organic micronutrients. Since P is taken up by plants from soil as phosphates, phosphate fertilizers are applied on fields to support the P-supply for crops. Today, shrinking global P-resources demand a reduction in the application of P-containing fertilizers, but knowledge about possible effects of a reduced phosphate-availability in soils on the quality of maize grains is lacking. Thus, it was hypothesized that a reduced phosphate-availability in soil influences the concentrations of dietary organic compounds (phenolics, fatty acids, carotenoids, and tocochromanols) in grains of maize during cultivation. Moreover, concentration differences in the P-storage form phytic acid in maize grains may impact the oxidative stability of these organic compounds during processing and digestion. Fertilizer experiments with maize hybrids were conducted at study sites with low to high phosphate concentrations in soil (1.6 to 20.6 mg CAL-P/100 g soil) in Germany. GC-MS or HPLC-(MS) analyses of the ground maize grains revealed the identity of fatty acids, insoluble (mostly diferulic and triferulic acids) and soluble (poly)phenols, carotenoids, and tocochromanols. The concentrations of these (poly)phenols, carotenoids, and tocochromanols as well as the fatty acid composition in the grains of the maize plants grown with or without phosphate fertilizer were not significantly ($p < 0.05$) different. Interaction effects between phosphate application and the locations on the fatty acid composition as well as on carotenoids and tocochromanols were considered as insignificant, concluding that a reduction in phosphate fertilization could be implemented on most fields in Germany when only considering these dietary compounds. Lastly, the influence of phytic acid on oxidation processes in maize during processing of porridge and in vitro digestion was examined. Porridges were prepared from maize flour containing either high phytic acid concentration or low phytic acid concentration supplemented with or without phytate. The porridges were digested using a human in vitro digestion model, resulting in a decrease in tocochromanols, carotenoids and unsaturated fatty acids. Oxidation products (α-tocopherylquinone, malondialdehyde) were formed in all samples, implying that phytic acid addition did not show the expected protective effect. The addition of phytate evoked a significant reduction in the micellarization efficiency of most carotenoids. Thus, the knowledge about phytic acid as antinutrient was extended.

Zusammenfassung

Phosphor (P) ist ein Element, das essenziell für lebende Organismen und an Phosphorylierungsreaktionen beteiligt ist. Letztere finden beispielsweise bei der Biosynthese einiger organischer Mikronährstoffe statt. Da P von Pflanzen als Phosphate aus dem Boden aufgenommen werden, setzen Landwirte Phosphatdünger ein, um eine ausreichende P-Versorgung der Nutzpflanzen zu gewährleisten. Sinkende globale Phosphorressourcen erfordern allerdings eine Reduktion der Anwendung von Phosphatdüngern, jedoch ist bisher nicht ausreichend bekannt, welche Auswirkung eine verringerte Phosphatverfügbarkeit auf die Qualität von Maiskörnern hat. Daher wurde die Hypothese aufgestellt, dass eine reduzierte Phosphatverfügbarkeit im Boden einen Einfluss auf die Biosynthese und Konzentrationen von ernährungsrelevanten Inhaltsstoffen (Phenole, Fettsäuren, Carotinoide, und Tocochromanole) in Maiskörnern während des Wachstums hat. Des Weiteren könnten unterschiedliche Konzentrationen in der P-Speicherform Phytinsäure einen Einfluss auf die oxidative Stabilität dieser organischen Substanzen während der Herstellung und des Verdaus von maisbasierten Lebensmitteln haben.

Düngemittelexperimente mit Hybridmaissorten wurde an Standorten mit niedrigen bis hohen P-Gehalten im Boden (1.6 bis 20.6 mg CAL-P/100 g Boden) in Deutschland durchgeführt. Mittels GC-MS oder HPLC-(MS) wurden Fettsäuren, unlösliche (Diferula- und Triferulasäuren) und lösliche (Poly)phenole, Carotinoide und Tocochromanole in den vermahlenen Maiskörnern identifiziert. Maispflanzen angebaut mit Phosphatdünger zeigten keine statistisch ($p < 0.05$) signifikanten Unterschiede der (Poly)phenol-, Carotinoid-, und Tocochromanolkonzentrationen sowie der Fettsäure-zusammensetzung in den Maiskörnern im Vergleich zu Kontrollproben. Interaktionseffekte zwischen der Phosphatdüngung und den Standorten auf die Profile von Fettsäuren, Carotinoiden und Tocochromanolen waren nicht signifikant. Daraus resultiert, dass eine Reduktion von Phosphatdüngern an den meisten Standorten in Deutschland möglich wäre, ohne die Gehalte dieser organischen Mikronährstoffe in Mais negativ zu beeinflussen.

Zuletzt wurde der mögliche Einfluss von Phytinsäure auf Oxidationsprozesse in Mais während der Verarbeitung und des in vitro Verdaus von Maisbrei untersucht. Maisbreie wurde aus Maismehl mit hohen Phytinsäuregehalten oder niedrigen Phytin-säuregehalten mit oder ohne zugesetztem Phytat hergestellt. Die Maisbreie wurden

mittels eines humanen in vitro Verdauungsmodells verdaut. Kochen und der darauffolgende Verdau verringerten Tocochromanole, Carotinoide und ungesättigte Fettsäuren. Oxidationsprodukte (α-Tocopherylchinon, Malondialdehyd) wurden in den Proben gebildet, welche den fehlenden Schutzmechanismus durch die zugesetzte Phytinsäure untermauern. Durch die Zugabe von Phytat wurde eine signifikant niedrigere Mizellierungseffizienz der meisten Carotinoiden beobachtet. Dadurch wurden weitere Erkenntnisse über Phytinsäure als Antinährstoff gewonnen.

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Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
$Ca_3(PO_4)_2$	Tricalcium phosphate
CAL	Calcium acetate-lactate
CD36	Cluster determinant 36
DAD	Diode array detection/detector
DNA	Deoxyribonucleic acid
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
ESI	Electrospray ionization
FAME	Fatty acid methyl esters
FID	Flame ionization detector
FLD	Fluorescence detection/detector
GC	Gas chromatography
GDD	Growing degree days
HM	High phytic acid maize
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
LM	Low phytic acid maize
LMS	Low phytic acid maize supplemented with phytate
$MS^{(n)}$	(Multiple-stage) mass spectrometry
MUFA	Monounsaturated fatty acids
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NIRS	Near-infrared spectroscopy
P	Phosphorus
P0	Cultivated without phosphate fertilizer
P44	Fertilized with 44 kg P/ha
PUFA	Polyunsaturated fatty acids

r	Correlation coefficient
SIM	Selected ion monitoring
SR-B1	Scavenger receptor class B type I
TPT	Triose-phosphate/phosphate translocator
TSP	Triple superphosphate
UV/vis	Ultraviolet/visible light (detection)
VDLUFA	Association of German Agricultural Analytic and Research Institutes

Chapter 1

Introduction

1 Phosphorus

Phosphorus (P) is a crucial element for living organisms, including humans, animals, and plants (Müller & Zhang, 2019). The discovery of the element P has been attributed to the German alchemist Henning Brand in the 17th century (Breger, 1987). Intending to find the philosopher´s stone, he obtained a white powder with self-illuminating properties after heating concentrated urine with sand. The term phosphorus, deriving from the Ancient Greek word φωσφόρος meaning carrier of light, was chosen to describe the element based on the observed luminescence (Föllmi, 1996). In the 18th century, the German scientist Justus von Liebig described in an article that mineral compounds including phosphorus-containing substances in soil are substantial for plant nutrition (Liebig, 1851). In 1908, Arthur Harden and William John Young experimentally confirmed the essential role of P as phosphate in biological systems. They reported that the rate of alcoholic fermentation of glucose by yeast is temporarily accelerated by the addition of phosphate (Harden et al., 1908). Today, the global application of P as phosphates is mainly as mineral fertilizer with nearly 80%, followed by the use as detergents with 12%, animal feedstuff with 5%, and minor applications (Steen, 1998).

1.1 Why is phosphorus essential to life?

1.1.1 Occurrence of phosphorus in nature

The average concentration of P in the earth crust is one g per kg (Stewart et al., 2015). Due to its high reactivity, P naturally occurs in the quinquevalent oxidation state (Larsen, 1967). In soils, P occurs in its inorganic and organic forms (Jantamenchai et al., 2022). The latter includes phosphonates and phosphate esters such as inositol phosphates, deoxyribonucleic acid (DNA), and break-down products of phospholipids (McLaren et al., 2017; Missong et al., 2016; Turner et al., 2007; Vestergren et al., 2012). In plants, P is present at concentrations of up to 0.2% of their dry weight (DW) (Schachtman et al., 1998). Sugar phosphates, phospholipids, inositol phosphates, nucleotides, and cofactors were identified in plant tissues (Bieleski, 1968; Ding et al.,

2017; Nieman & Clark, 1976). During ripening, the concentration of P increases in maize grains (Modi & Asanzi, 2008). At harvest maturity, the average total P-concentration in maize is 3.2 g P/kg DW (Rodehutscord et al., 2016). The highest proportion of total P in cereals is attributed to *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, commonly known as phytic acid (Raboy et al., 2000; Rodehutscord et al., 2016). In maize, approximately 88% of the total phytate is concentrated in the germ. These reservoirs supply the seedlings with P during early germination (White & Veneklaas, 2012).

Unlike plants, the majority of the total P in humans is located in their skeleton (85%) followed by soft tissues (14%) and extracellular body fluids (1%) (Amanzadeh & Reilly, 2006). In addition, monophosphates including intracellular and extracellular phosphate salts, diphosphates such as nicotinamide adenine dinucleotide, and triphosphates mainly as adenosine triphosphate (ATP) are common molecules found in humans (Ren et al., 2015). In summary, phosphates are ubiquitously present as intermediates of biochemical reactions or metabolites in nature (Westheimer, 1987).

1.1.2 Biological relevance of phosphorus and phytate for plants and humans

Phosphates are involved in important metabolic reactions, especially for the biosynthesis of DNA and ribonucleic acid, synthesis of phospholipids as membrane components, energy transfer as ATP, and phosphorylation reactions in signaling cascades (Brawerman & Chargaff, 1954; Exterkate et al., 2018; Voelkl et al., 2021; Wieland & Bäuerlein, 1968). In photosynthesis, the triose-phosphate/phosphate translocator (TPT) of the inner membrane of the chloroplasts mediates the counter-exchange of triose-phosphate, derived from carbon dioxide assimilation, with phosphate (Heldt & Rapley, 1970; Lee et al., 2017; Stocking & Larson, 1969). Within the stroma of chloroplasts, P-deficiency decreases the concentration of orthophosphate to levels that inhibit the activity of ATP synthase, decreasing the levels of ATP in plants and, therefore, the carbon dioxide fixation (Carstensen et al., 2018). Strong responses to limited P were reported for maize with a sharp decrease by 68.3% for the yield per plant. Interestingly, the P concentrations in the maize grains were only slightly reduced (Li et al., 2021). The dependency of P also becomes evident in the plant fraction of soybean nodules, where limited access to P reduces symbiotic nitrogen fixation by impairing the oxidative phosphorylation and nitrogenase activity

(Sa & Israel, 1991). Another aspect is that phosphates are essential for tocochromanol synthesis, where phytyl pyrophosphate is required for the prenylation of homogentisic acid (Soll et al., 1980).

As mentioned earlier, phosphate is also stored as inositol phosphate in grains and is released by enzymatic hydrolysis during germination (Matheson & Strother, 1969). Therefore, intrinsic phytase (*myo*-inositol-hexa*kis*phosphate phosphohydrolase) catalyzes the dephosphorylation of phytate and minor inositol phosphates in grains and its activity increases with proceeding germination (Laboure et al., 1993). As a consequence of hydrolysis, phytate is degraded while phosphates are released and further translocated into the developing seedling (Eastwood & Laidman, 1971). From a nutritional point of view, phytate is commonly labeled as an antinutrient given the six phosphate groups of its structure that can strongly chelate minerals (Urbano et al., 2000).

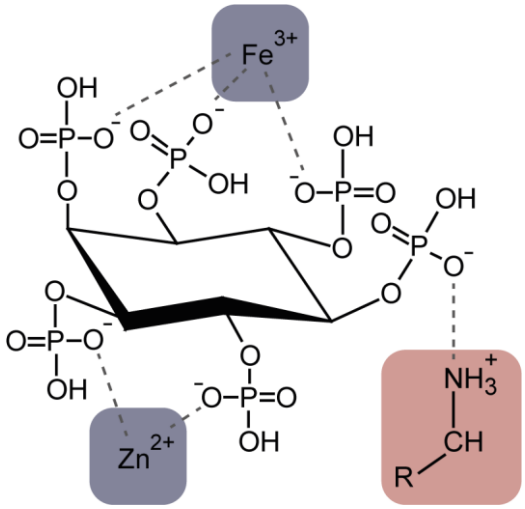


Figure 1: *Myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate can bind bivalent and trivalent cations as well as compounds carrying an amino group (for instance proteins, peptides, amino acids). Metal ions are highlighted in blue and amino-group carrying compounds are marked in red. The figure was modified from Yu et al. (2012) and Agranoff (2009).

Even though some authors reported endogenous phytase activity in intestinal mucosa of humans (Bitar & Reinhold, 1972), human trials revealed an impaired absorption of iron up to 82% when 250 mg of sodium phytate were added to the test meal (Hallberg et al., 1989). Phytic acid also reduced the absorption of other dietary minerals such as manganese and zinc (Bohn et al., 2004; Turnlund et al., 1984). Within the pH range of 2.5 and 3.8, soy protein builds aggregates with phytate, which may decrease the

protein digestibility (Yu et al., 2012). An example of phytate binding minerals and proteins is summarized in Figure 1.

Phytic acid can also inhibit iron-mediated oxidative reactions (Fenton reaction) and reduces ascorbic acid degradation (Empson et al., 1991). It was further proposed that iron-phytate-complexes do not possess a reactive coordination site that can produce hydroxyl radicals, thus, being more effective than ethylenediaminetetraacetic acid (EDTA) in preventing the hydroxyl radical formation (Graf et al., 1984).

In humans, phosphates found in the plasma contribute to 1.5% of the total blood buffer capacity to maintain physiological pH (Ellison et al., 1958). On the other hand, excessive intake of phosphates can cause hyperphosphatemia (> 1.5 mmol/L) in humans, which increases the risk for cardiovascular diseases (McGovern et al., 2013).

1.2 The phosphate cycle in agriculture

1.2.1 Phosphate-availability and its testing methods

One of the most immediately available forms of P for plants are orthophosphates (Coventry et al., 2001). Within this group, it has been suggested that the monovalent form of inorganic P ($H_2PO_4^-$) is preferentially taken up by higher plants (Schachtman et al., 1998). However, the concentration of inorganic P in the soil solution is strongly dependent on adsorption-desorption mechanisms, as well as dissolution equilibria. These parameters are affected by soil pH, presence of cations such as calcium, aluminum, and iron, and the presence of organic ligands (Hinsinger, 2001).

Even though some crops can adapt to low P-availability in soils, P-containing fertilizers are applied on fields to improve its availability and stabilize their yields of production (Vance et al., 2003). For this purpose, chemical fertilizers derived from mined rock phosphate are produced and commercialized (Schoumans et al., 2015). Another option to increase P-pools in arable lands is the application of manure, which is a by-product of life-stock farming (Komiyama et al., 2014).

In order to capture P-fractions in soils, different P-extraction methods have been developed. These methods mainly differ in the composition of the extracting agent. The widely applied Olson method is based on the removal of calcium, as calcium carbonate, to increase the solubility of calcium phosphates in alkaline, neutral, and calcareous soils using an alkaline sodium hydrogencarbonate solution (Olsen et al.,

1954). The P-concentration in soil determined by the Ohlson method is comparable with the concentration of orthophosphates (Coventry et al., 2001). Another important extracting agent named Mehlich 3 consists of a combined solution of acetic acid, nitric acid, ammonium fluoride, ammonium nitrate, and EDTA. The Mehlich 3 solution is used to extract P from slightly acidic soils (Mehlich, 1984). The calcium acetate-lactate (CAL) method is based on an extracting agent composed of calcium acetate, calcium lactate, and acetic acid (Schüller, 1969). The resulting CAL-P value is a common parameter used to indicate recommendations on P-fertilization in Germany. Following this CAL-P value, soils are classified from very high P concentration (> 12 mg CAL-P/100 g soil) to very low P-concentration in soil (< 1.5 mg CAL-P/100 g soil), for regions with an annual rainfall greater than 550 mm/year, according to the Association of German Agricultural Analytic and Research Institutes (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2018).

1.2.2 Uptake and recovery of phosphate

In soil, P is mainly transported to the root system by diffusion (Olsen et al., 1962). From the roots, P is transported to the xylem and distributed to the younger leaves (Hamburger et al., 2002; Mimura et al., 1996). As a consequence, the P-concentration in the leaves and stems increases during plant development (Ciampitti et al., 2013). Until physiological maturity (R6 stage), P accumulates in the grains and in the whole plant of maize (Woli et al., 2018). Therefore, harvesting crops removes P from the fields and 70% of the global harvested P comes from cereals (Liu et al., 2008). In the case of maize, the crop is further utilized for bioethanol and biogas production or processed for animal or human consumption (Gulati et al., 1996; Ranum et al., 2014; Schulz et al., 2018).

In contrast to plants, phosphate in animals and humans is absorbed in the small intestine (Danisi & Straub, 1980; McHardy & Parsons, 1956; Walton & Gray, 1979). In the body, phosphates are taken up from the intestinal lumen by passive diffusion or by sodium-dependent phosphate co-transport (Hilfiker et al., 1998; Sabbagh et al., 2011). Within the body, phosphate is distributed in extracellular fluids, used for remodeling of bones, and partly enters the tubular fluids in the kidney, where phosphate can also be reabsorbed (Berndt et al., 2005). Approximately 200 mg of P per day are secreted back to the intestine as a component of digestive juices (Berndt et al., 2005). Excess of P in

the body is excreted by the urine (Boyd et al., 1930). It was estimated that P from human urine makes up to 1.68 million tons, which can cover up to 22% of the worldwide P-demand (Mihelcic et al., 2011). Since then, different recovery strategies of P from urine have been developed, including adsorption or crystallization processes among others (Guan et al., 2020; Le et al., 2020). In the same line, struvite (magnesium ammonium phosphate), precipitated from waste-water, is a promising P-source for fertilizers. However, P-uptake from struvite by plants is only about 26% (Talboys et al., 2016). When applying fresh beef cattle manure on croplands, higher P and nitrogen in surface runoffs were observed, compared to composted manure (Miller et al., 2006). A summary of the described phosphate cycle in given in Figure 2.

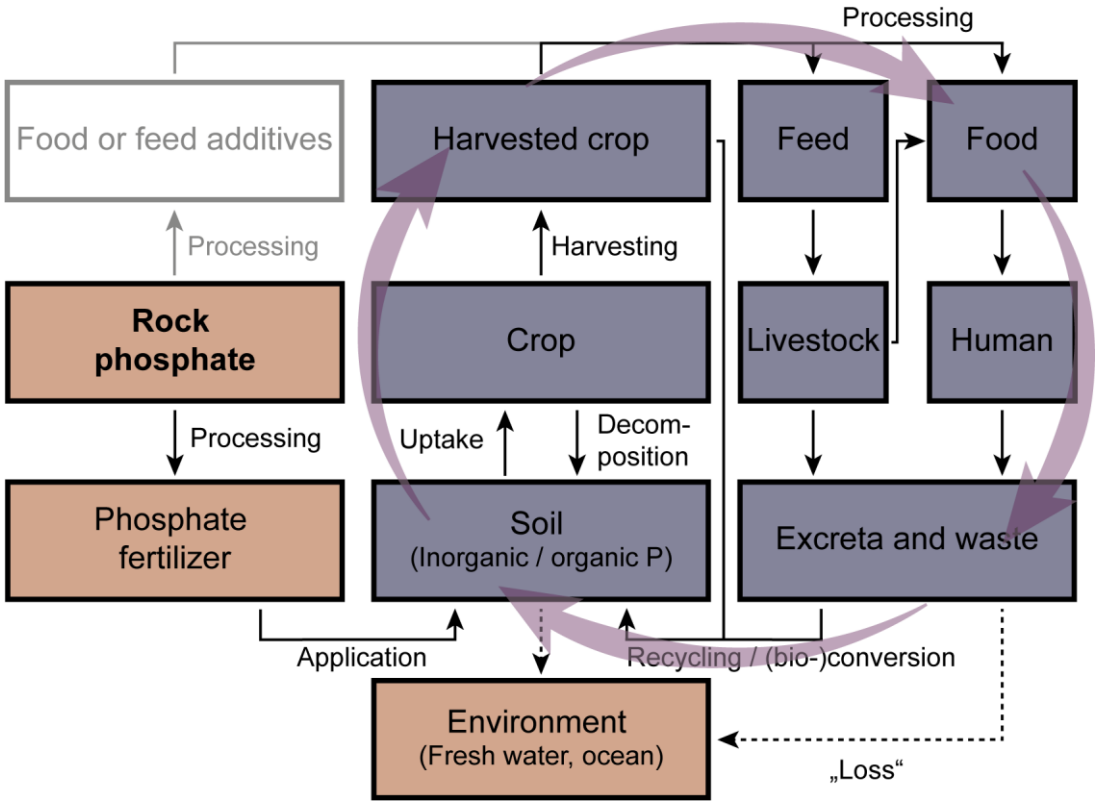


Figure 2: A simplified scheme of the global phosphate cycle in agriculture. Phosphate flows are represented by black arrows in the figure. Inorganic phosphate fertilizers are produced from mined rock phosphate and applied on fields. Crops take up plant-available phosphate from the soil. Then, phosphates are partly removed from the field as constituents of the harvested crop, while crop residues containing phosphate remain on the field and decompose. Harvested crops are subsequently used for animal and human consumption or biomass conversion. Edible products of livestock farming are also consumed as food by humans. Phosphates from excreta and by-products from human consumption and livestock production are either recycled, reapplied as fertilizer on the field or enter the environment (rivers, lakes, oceans) as a part of waste-water. Soil erosion and runoffs also contribute to a loss of phosphates into the environment. Based on (Müller & Zhang, 2019; Smil, 2000).

1.2.3 Reasons for reducing phosphate input in agricultural systems

Today, three major issues that are connected with the unbalanced global phosphate cycle are arising. First, the sum of the yearly global input of P (14.2 and 9.6 Tg of P from fertilizer and manure, respectively) exceeds the P-removal by crops (12.3 Tg of P) (MacDonald et al., 2011). This is partly explained by the low efficiency of cereal crops for P-uptake, with an average value of 16% as determined by the weight difference method (Dhillon et al., 2017). Thus, long-term P-application can cause an accumulation of P in soil, which has been demonstrated for example by the significant increase in Olsen-P concentrations (Xi et al., 2016; Zhang et al., 2020). As a consequence, an increase in the risk for eutrophication is expected due to excessive losses of nutrients including P from soils into water bodies (Tilman et al., 2001). Given these reasons, one of the goals of the “Farm to Fork Strategy” of the European Commission is to reduce the application of fertilizers by at least 20% and to avoid nutrient losses in soils by up to 50% by 2030 (European Commission, 2020, 2021).

Second, elevated concentrations of heavy metals have been found in mineral fertilizers such as rock phosphate (Giuffréde López Carnelo et al., 1997; Williams & David, 1973), and therefore may pose a risk for soil contamination. In another study, the higher concentrations of uranium found in arable soils compared to grassland were justified by the higher input of mineral fertilizers in the first ones (Bigalke et al., 2017).

Third, as a non-renewable resource, it is projected that global P-reservoirs used for the production of P-fertilizers from rock phosphate will be depleted within a century. This is especially important considering that P-demand is projected to increase and to reach its peak in 2030 (Cordell et al., 2009). P-resources are unevenly distributed on earth with approximately 77% of the currently established P-reserves located in Morocco (Cooper et al., 2011).

In order to balance crop productivity and provide nutritious foods for future generations, the global phosphate cycle in agriculture needs to be adapted and the effects of phosphate limitation on the crops, especially of maize as a multi-purpose crop, need to be better understood (Müller & Zhang, 2019). When assessing P-application rates for crops, nutritional quality should be taken into account, because it has already been observed that P-application decrease mineral micronutrients such as zinc, as it was found in shoots of summer maize (Zhang et al., 2017).

2 Dietary lipids and minor organic compounds in maize

Maize is one of the main produced cereals on the globe, with a total production volume of 1.16 billion metric tons in 2020. The three main producers of maize are the United States (360.25 million metric tons), China (260.67 million metric tons), and Brazil (103.96 million metric tons). Maize and maize products are mainly consumed in African countries with an annual consumption of 43.2 kg per person (average consumption over the years 2010-2019) (FAOSTAT, 2022). By 2050, a threefold increase in the demand for maize is anticipated in Sub-Saharan countries, where maize is consumed as whole-grain foods, wet-ground dish, porridges, bread, snacks, and beverages (Ekpa et al., 2018). From a nutritional aspect, maize grains mainly consist of starch (78.92% to 85.79%) followed by protein (8.35% to 13.88%), fat (6.02% to 3.95%) and ash (1.09% to 1.74%), and their composition can be modified by breeding (Hopkins, 1899). Maize kernels also exhibit a remarkable diversity in substantive minor lipophilic (vitamin E, provitamin A carotenoids) and hydrophilic (B-vitamins) nutrients, essential minerals, as well as (poly)phenols (flavonoids, phenolic acids) (Adom & Liu, 2002; Muzhingi et al., 2008; Teas, 1954; Urias-Lugo et al., 2015; Xie et al., 2017). For the present study, (unsaturated) fatty acids, hydroxycinnamic acid derivatives, carotenoids and tocochromanols were selected, because they are essential micronutrients for humans and require P for their biosynthesis or they are involved in stress responses of plants and are associated with health benefits for humans.

2.1 Fatty acids

2.1.1 Structure, biosynthesis, and occurrence of fatty acids

Fatty acids are chemically defined as carboxylic aliphatic acids with the general molecular formula of $H(CH_2)_nCOOH$ and a carbon chain length of 1 to 40 for simple saturated fatty acids (Brondz, 2005). Besides saturated fatty acids, unsaturated fatty acids have been predominantly identified in vegetable oils which are classified into monounsaturated fatty acids (MUFA), with one double bond, or polyunsaturated fatty acids (PUFA), with at least two double bonds in the chain (Orsavova et al., 2015). In maize germs, fatty acids mostly occur in their esterified form as triacylglycerides, making up to 93.3% of the total lipids, while free fatty acids account for only 0.6% (Weber, 1979). Main fatty acids in maize include palmitic (16:0), stearic (18:0), oleic

(18:1), linoleic (18:2), and linolenic acid (18:3), accounting for 98.4% of the total fatty acid contents (Li et al., 2013). In addition, glycolipids (mono- and digalactosyldiacylglycerol) and phospholipids (phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine) with saturated and unsaturated fatty acid moieties have been identified in maize (Rizov & Doulis, 2000).

In plants, biosynthesis of fatty acids takes place in plastids (Hawke et al., 1974; Kannangara et al., 1971). Briefly, saturated long-chain fatty acids are *de novo* synthesized from acetyl coenzyme A by acetyl-coenzyme A carboxylase and fatty acid synthetase complexes (Harwood, 1996). For this process, large amounts of ATP (547 nmol ATP per hour and embryo) and the reduced form of nicotinamide adenine dinucleotide phosphate (547 nmol NADPH per hour and embryo) as reducing equivalent are required, as it was reported for the biosynthesis of fatty acids in plastids of developing maize embryos (Paula Alonso et al., 2010). These high needs of ATP and NADPH underline the possible dependence on the process of P-availability. Unsaturated fatty acids are subsequently formed catalyzed by fatty acid desaturases and their content and regulation is important for the maintenance of membrane fluidity (Harris & James, 1965; Zhao et al., 2019).

At a later stage, fatty acids are transferred from the plastids to the endoplasmic reticulum as acyl esters. In this organelle, diacylglycerol is assembled via the Kennedy pathway (Chapman & Ohlrogge, 2012). Diacylglycerol is further converted into membrane glycerolipids (for instance phosphatidylcholine by the phosphocholine transferase) or into triacylglycerides catalyzed by the diacylglycerol acyltransferase (Cao & Huang, 1986; Gibellini & Smith, 2010). The latter are stored in seeds as lipid bodies surrounded by a layer composed of phospholipids and embedded oleosins (Bergfeld et al., 1978; Huang, 1992). Especially during grain development triacylglycerols strongly accumulate in the germ, reaching a maximum concentration between 36 to 41 days after pollination (Tan & Morrison, 1979).

2.1.2 Biological function and importance of fatty acids for human nutrition

One of the main functions of saturated fatty acids in plant seeds is the breakdown into acetyl units by β-oxidation during germination (Stumpf & Barber, 1956). The resulting acetyl units are then used for sugar synthesis (Canvin & Beevers, 1961). Furthermore, in maize root tips submitted to sugar starvation, an increase in the activities of β-

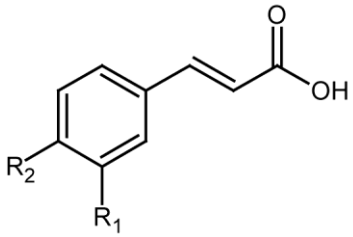
oxidation enzymes was observed, probably as response of the plant tissue, shifting from carbohydrates to lipids as the main respiratory substrates (Dieuaide et al., 1992). In addition to the function as energy reserves, fatty acid esters serve as membrane components, represent parts of epicuticular layers as waxes which may support the protection of the leaves against dehydration, and are involved in stress signal transduction (Bourgault et al., 2020; Harwood, 1996; Klimecka et al., 2011; Murphy & Parker, 1984; Slocombe et al., 2009).

In contrast to plants, mammals are not able to biosynthesize polyunsaturated fatty acids with the first double bound at the *n*-6 (e.g. linoleic acid) or *n*-3 position (e.g. α-linoleic acid), and need to obtain these essential fatty acids from their diet (McCowen & Bistrian, 2005). The essentiality of *n*-3 and *n*-6 fatty acids as part of the diet was first shown by feeding rats with a diet absent in fats, which resulted in caudal necrosis and drastic underweight (Burr & Burr, 1929). Apart from that, positive health effects after the intake of polyunsaturated fatty acid containing foods have been reported such as a reduction in diastolic blood pressure for patients with hypertension (Rao et al., 1981), and a reduced risk for fatal ischemic heart disease (Hu et al., 1999), among others. In addition, an association between a frequent intake of linoleic acid and a lower risk for type 2 diabetes was found for men below 65 years (van Dam et al., 2002). On the other hand, polyunsaturated fatty acids are prone to oxidation (rancidity) limiting the shelf-life of foods rich in these compounds and leading to the generation of undesired oxidation products that have been associated with inflammation (Porter et al., 1981; Raphael & Sordillo, 2013). As a countermeasure, α-tocopherol was reported to protect polyunsaturated fatty acids from lipid peroxidation due to its radical scavenging ability (Terrasa et al., 2009).

2.2 Hydroxycinnamic acid derivatives

2.2.1 Structure, biosynthesis, and occurrence of hydroxycinnamic acid derivatives

Hydroxycinnamic acid derivatives, for instance *p*-coumaric acid and ferulic acid (Figure 3), are phenolic compounds ubiquitously present in most plant-based foods (Herrmann & Nagel, 1989). These compounds are characterized by a phenylpropanoid C_6-C_3 skeleton with an unsaturated side chain (El-Seedi et al., 2012).



Hydroxycinnamic acid derivative	R_1	R_2
p-Coumaric acid	H	OH
Caffeic acid	OH	OH
Ferulic acid	OCH_3	OH

Figure 3: Examples of hydroxycinnamic acid derivatives identified in foods. Modified from El-Seedi et al. (2012).

In general, it is assumed that hydroxycinnamic acids are biosynthesized from the aromatic amino acids L-tyrosine and L-phenylalanine originating from the shikimate pathway (Marchiosi et al., 2020). After deamination of tyrosine or phenylalanine, cinnamic acid and *p*-coumaric acid are formed which are further modified by hydroxylation and methylation resulting in the derivatives ferulic acid and caffeic acid (Heleno et al., 2015). In maize grains, hydroxycinnamic acid derivatives are further divided into free (soluble) and bound (insoluble) phenolics, according to the applied sample preparations (Adom & Liu, 2002). Insoluble hydroxycinnamic acid derivatives result from the ester or ether linkages of ferulic acid or *p*-coumaric acid to cell-wall components which can only be hydrolyzed under strong alkaline and acidic conditions (Sun et al., 2001). On average, maize grains contain higher amounts of free (0.92 µmol/100 g) and bound ferulic acid (896.27 µmol/100 g) than wheat, oats, and rice (Adom & Liu, 2002). Within maize grains, the concentration of total free and bound phenolics is higher in the pericarp than the germ and endosperm (Das & Singh, 2016). The concentration of phenylpropanoid secondary metabolites is significantly increased in leaves of maize seedlings grown in nutrient solutions with low nitrogen (0.15 mmol/L KNO_3) or low P (0.1 mmol/L KH_2PO_4) concentration (Schlüter et al., 2013). In addition, highly complex dehydrodimers and trimers of ferulic acid have been identified in maize bran (Bunzel et al., 2004, 2005). Another special group of metabolites found in maize kernels are phenolamides, a group of secondary metabolites whose structure results from the amide-linkage of phenolic acids with aliphatic or aromatic amines (Roumani et al., 2020; Wen et al., 2014).

2.2.2 Biological function and importance of hydroxycinnamic acid derivatives for human nutrition

In maize, hydroxycinnamic acid derivatives, mainly ferulic acid, are associated with the protection against pests, fungal diseases, and stress responses (Alvarez et al., 2008; Santiago et al., 2007; Soujanya et al., 2021). For instance, xylans cross-linked by diferulates partially inhibit cell-wall hydrolyses by fungal enzymes in maize (Grabber et al., 1998). Furthermore, cinnamic acid can mitigate the negative effects of reactive oxygen species (ROS), by increasing the activity of ROS scavenging enzymes in maize plants grown under abiotic stress such as salt stress (Singh et al., 2013). A strong correlation (r = 0.999) between bound ferulic acid and total antioxidant activity has been described in the literature (Adom & Liu, 2002).

Today, little information about the absorption of hydroxycinnamic acid derivatives from maize and maize-based products by humans is available. In a rat study, the bioavailability of ferulic, *p*-coumaric, and diferulic acid after ingestion of corn bran was very low or undetectable (Zhao et al., 2005). Only a small fraction of feruloyl groups, which are mainly esterified in fiber, are released during gastric and small intestinal digestion in humans and about 95% of the esterified feruloyl groups were fermented in the large intestine by bacteria (Kroon et al., 1997). During absorption, phenolic compounds are metabolized for instance by methylation or glucuronidation in the small intestine and in the liver which may facilitates their elimination by the biliary or urinary pathway (Manach et al., 2004).

Despite their low bioavailability, hydroxycinnamic acid derivatives and their conjugated phenolic amides in corn bran are associated with an anti-inflammatory activity, for instance by inhibiting inducible nitric oxide synthase expression in macrophages (Kim et al., 2012). The administration of ferulic acid to rats through their diet produced insulin sensitivity in rats and is partially effective against hypertension (Senaphan et al., 2015).

2.3 Carotenoids

2.3.1 Structure, biosynthesis, and occurrence of carotenoids

Carotenoids are lipophilic pigments mainly biosynthesized by plants and other photosynthetic organisms (Stahl & Sies, 2005). Even though humans and animals are not able to synthesize these compounds *de novo*, they are not considered essential

nutrients, since carotenoids are not directly involved in a vital metabolic pathway (Arunkumar et al., 2018; Hammond & Renzi, 2013). The chemical structure of carotenoids is mostly based on a C_{40} structure with a conjugated polyene chain consisting of eight isoprene units (tetraterpene) (Srivastava, 2021). Chemically, carotenoids are classified into carotenes, which are composed of pure hydrocarbons such as lycopene, α- and β-carotene, and the more polar xanthophylls, for instance α- and β-cryptoxanthin, lutein, and zeaxanthin, carrying at least one oxygen in their structure (Stahl & Sies, 2005). Due to their large number of conjugated double bonds, carotenoids have absorption maxima in the visible range, for instance lycopene with absorption maxima at 502.5, 471.0, and 444.0 nm determined by spectroscopy in hexane (Takehara et al., 2014). In addition, carotenoids are susceptible against oxygen, heat, and irradiation which may lead to degradation and (*E*/*Z*)-isomerization as observed for lutein and zeaxanthin (Li et al., 2014; Zhang et al., 2016). Thus, proper post-harvest management is essential to minimize carotenoid degradation in whole maize grains and flours. Adequate storage conditions include low temperature, low relative humidity, and light exclusion (Awoyale et al., 2018; Ortiz et al., 2016).

In maize plastids, the biosynthesis of carotenoids is based on the isoprenoid pathway (Zhang et al., 2019). Briefly, one molecule of dimethylallyl pyrophosphate derived from the methylerythritol-4-phosphate (MEP) pathway is condensed with three molecules of isopentenyl pyrophosphate, giving as initial precursor the molecule geranylgeranyl pyrophosphate (Cervantes-Cervantes et al., 2006). The dimerization of geranylgeranyl pyrophosphate yields phytoene, a reaction that is catalyzed by the phytoene synthase (Lütke-Brinkhaus et al., 1982). After desaturation and isomerization reactions, lycopene is synthesized (Bartley et al., 1999; Chen et al., 2010; Isaacson et al., 2002). Cyclization at both ends of the lycopene molecule leads to the formation of β-carotene by action of lycopene β-cyclase and α-carotene by action of lycopene ε- and lycopene β-cyclase (Cazzonelli, 2011). Further hydroxylation at C-3 and/or C-3' positions of α- and β-carotene generates xanthophylls (Tian et al., 2003; Walton et al., 1969). The hydroxylation of α-carotene leads to lutein while zeaxanthin derives from β-carotene via β-cryptoxanthin (Kim & DellaPenna, 2006; Sun et al., 1996). Among cereals, carotenoids, mainly lutein, have predominantly been identified in grains of maize, sorghum, wheat and other *Triticum* species (Owens et al., 2014; Przybylska-Balcerek et al., 2019; Ziegler et al., 2015). Important sources of carotenoids are also chloroplast-rich green-leafy vegetables, fruits, and animals such as fish, which can accumulate

carotenoids in their muscle tissues (Aman et al., 2005; Gowele et al., 2019; Schweiggert et al., 2011; Steingass et al., 2020; Torrissen, 1989).

An elevated dose of nitrogen fertilization significantly ($p < 0.05$) increases the concentrations of lutein and zeaxanthin in maize grains without significant effects in the concentrations of β-carotene and β-cryptoxanthin (Giordano et al., 2018). Stress conditions such as elevated sodium chloride concentrations in soils lead to an increased expression of phytoene synthase genes, which is associated with an increase in carotenoid concentrations in *Arabidopsis* (Ruiz-Sola et al., 2014). Thus, carotenoid concentrations in maize grains may vary depending on agricultural management and abiotic stress conditions.

2.3.2 Biological function and importance of carotenoids for human nutrition

Carotenoids fulfill several important functions for plants, mainly due to their light absorbing properties (Maoka, 2020). In chromoplasts, carotenoids can attract animals through their yellowish to orange color for seed dispersion (Lopez-Juez & Pyke, 2005). As pigments of natural origin, carotenoids, in particular lutein and β-carotene, are also utilized as food and beverage colorants (Giménez et al., 2015). In chloroplasts, individual carotenoids contribute to the proper functioning of photosynthesis by absorbing light and transferring energy to chlorophylls (Marin et al., 2011). In addition, through a quenching mechanism, carotenoids can avoid damage to the photosynthesis apparatus (Mathis et al., 1979; Sistrom et al., 1956). β-Carotene reacts with peroxyl radicals at low partial pressures of oxygen (< 150 torr), which is found in most tissues under physiological conditions, and thereby can mitigate lipid peroxidation (Burton & Ingold, 1984). Cleaved carotenoid products are precursors of plant signaling molecules such as abscisic acid, which is involved in the stress-response of plants (Booker et al., 2004; Qin & Zeevaart, 1999; Tan et al., 1997).

For human health, several beneficial functions of carotenoids have been described including roles in eye and cardiovascular health, cognitive benefits, and the possibility of preventing certain types of cancer (Chew et al., 2014; Eggersdorfer & Wyss, 2018; Gajendragadkar et al., 2014; Grodstein et al., 2007; Wan et al., 2014). In 1919, after observing fat-soluble vitamin deficiency symptoms in rats fed white maize compared to yellow maize, it was assumed that the yellow plant pigment was associated with the normal growth and reproduction of the rats (Steenbock, 1919). Ten years later, it was

reported that a purified carotenoid-extract obtained from carrots had growth-enhancing effects on rats (v. Euler et al., 1929). Today, it is well established that these molecules are physiologically relevant because carotenoids possessing at least one β-ring are retinoid (vitamin A) precursor (Reboul, 2019). Examples of provitamin A carotenoids are summarized in Figure 4.

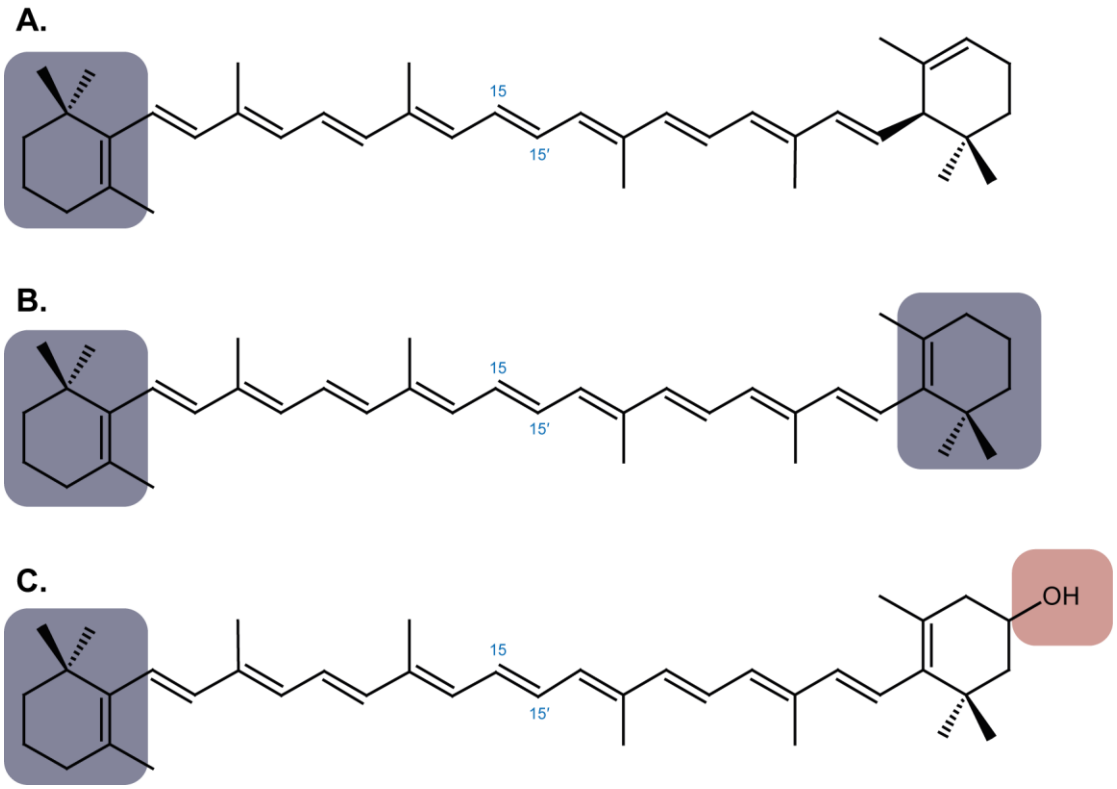


Figure 4: Chemical structures of α-carotene [A], β-carotene [B], and β-cryptoxanthin [C] functioning as provitamin A carotenoids. β-Rings (blue) and hydroxy group (red) are highlighted. The carbon numbers 15 and 15′ refer to the cleavage position for β-carotene 15-15′-oxygenase. The figure was modified from von Lintig (2020) and Diepenbrock et al. (2021).

Naturally, humans and animals obtain carotenoids from their foods (Arunkumar et al., 2018). The reduction in particle size of the food by mastication is a decisive step because it facilitates the release of carotenoids during digestion (Lemmens et al., 2010). Following ingestion, carotenoids are liberated from the food matrix and encapsulated in mixed micelles containing co-digested lipids and bile salts in the small intestine (Pérez-Gálvez et al., 2003; Salvia-Trujillo et al., 2017). Today it is still under debate whether carotenoids are absorbed by passive diffusion or by membrane transporters (Reboul, 2013). Two proteins, namely scavenger receptor class B type I (SR-B1) and cluster determinant 36 (CD36), have tentatively been identified as important biomolecules for the uptake mechanism of provitamin A carotenoids (Borel

et al., 2013). In the enterocyte, β-carotene is enzymatically cleaved into retinal by the β-carotene 15-15′-oxygenase (dela Seña et al., 2014). Retinal is then reduced to retinol and can be acylated into retinyl esters (O'Byrne et al., 2005; Reboul, 2013). In the postprandial state, carotenoids and retinyl esters are integrated into chylomicrons and transported through the lymphatic system (Nayak et al., 2001; Pérez-Gálvez et al., 2003). These chylomicrons are then mainly transported to the liver where retinyl esters accumulate (Blomhoff et al., 1982). Inadequate serum levels (< 0.7 µmol/L of vitamin A) can have severe health consequences such as night blindness in children and pregnant women (Black et al., 2013). Thus, maize resulting from provitamin A biofortification programs have recently been tested in nutritional intervention studies aiming at an improvement in the vitamin A status in humans (Gannon et al., 2014; Li et al., 2010). So far, consumption of maize meals prepared from biofortified maize (15 to 20 µg of β-carotene/g) has increased serum β-carotene levels in Zambian children aged four to eight years by 0.14 µmol/L compared to consumption of white maize (< 2 µg of β-carotene/g), but serum retinol concentrations have not improved (Palmer et al., 2016).

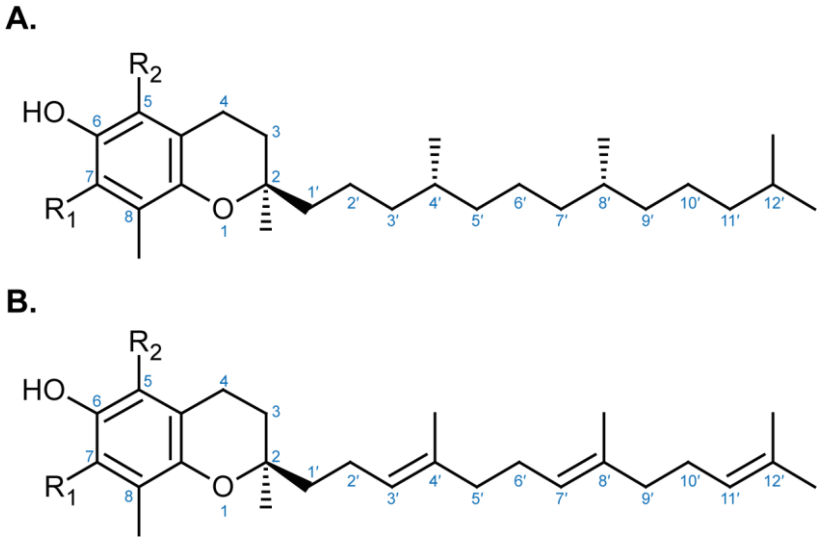
2.4 Tocochromanols

2.4.1 Structure, biosynthesis, and occurrence of tocochromanols

Tocochromanols are a group of organic compounds biosynthesized by photosynthetic organisms and represent a crucial dietary constituent in human and animal nutrition (Dörmann, 2007). The generic structure of tocochromanols consists of a methylated chromanol ring bound to a saturated or an unsaturated polyprenyl side chain (Falk & Munné-Bosch, 2010). Tocochromanols connected with a saturated side chain are designated tocopherols. The term tocotrienol is introduced for tocochromanols with double bonds positioned at carbons number 3′, 7′ and 11′, resulting in a threefold unsaturated polyprenyl chain (DellaPenna, 2005). The hydroxyl group of tocopherols can be esterified with fatty acids forming tocopheryl fatty acid esters, which have been identified in maize germ oil (Krauß et al., 2018).

The Greek prefixes α, β, γ, or δ refer to the position and quantity of the methyl groups at carbon numbers 5, 7, or 8 at the chromanol ring (IUPAC-IUB Commission on Biochemical Nomenclature (CBN), 1974). General structures of the four tocopherols and four tocotrienols are shown in Figure 5. Tocopherols, tocotrienols, and their

derivatives, resembling the biological activity of α-tocopherol, are also known under the trivial name “vitamin E” (Kamal-Eldin & Appelqvist, 1996).



Vitamin E congener	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 5: Chemical structures of tocopherols [A] and tocotrienols [B]. The latter are distinguished from tocopherols by their double bonds at carbon positions 3′, 7′ and, 11′. Carbon positions are marked blue. The figure was modified from Ricciarelli et al. (2002) and Krauß et al. (2018).

In plants such as maize, the biosynthesis of tocochromanols takes place in the plastids and requires prenyl diphosphates and homogentisic acid, derived from the catabolism of aromatic amino acids (Diepenbrock et al., 2017). The main step in the biosynthesis of tocopherols is the condensation of phytyl pyrophosphate, a reaction product of the non-mevalonate pathway, with homogentisic acid yielding 2-methyl-6-phytylbenzoquinol (Marshall et al., 1985; Soll et al., 1980; Soll & Schultz, 1980).

This reaction is catalyzed by the homogentisate phytyltransferase (Collakova & DellaPenna, 2001). In the case of tocotrienol synthesis, the first important reaction is catalyzed by a homogentisic acid geranylgeranyl transferase, which uses geranylgeranyl pyrophosphates and homogentisic acid as substrates (Cahoon et al., 2003). Further enzymatic methylations and a cyclization generate the naturally occurring α-, β-, γ-, δ-tocopherols and -tocotrienols (Cheng et al., 2003; Porfirova et al., 2002; Yusuf et al., 2010; Zhang et al., 2013). Tocochromanols have typically been

associated with membranes, where their hydrophobic side chain is positioned within the membrane and the polar chromanol ring is at the membrane surface (DellaPenna, 2005). α-Tocopherol prevails in photosynthetic tissue (Abbasi et al., 2007). In comparison to this, a larger variability in tocopherols and tocotrienols has been reported for maize grains (Xie et al., 2017).

In maize grains, γ-tocopherol had the highest concentrations among the analyzed tocochromanols (Goffman & Böhme, 2001). Analyses of a panel comprising 252 maize lines showed that γ-and δ-tocopherols were more abundant than their respective tocotrienols (Lipka et al., 2013). Representative plant sources containing high concentrations in tocochromanols are palm fruits (γ-tocopherol, α-tocopherol and -tocotrienol) as well as almonds and hazelnuts (mainly α-tocopherol) (Irías-Mata et al., 2017; Stuetz et al., 2017). Recently, the rarely investigated α- and γ-tocomonoenols have been identified in maize oil but their concentrations were not detectable after nine days under accelerated oxidation conditions (Alberdi-Cedeño et al., 2019).

Under high ozone concentrations, a drastic increase in the α-tocopherol concentration was observed in maize leaves, when compared to a control group not subjected to this artificial stress (Wedow et al., 2021). When grown under water stress, opposite results were reported in *Arabidopsis* mutants with a defective ascorbic acid metabolism in their chloroplasts, resulting in a decrease in the α-tocopherol concentrations (Munné-Bosch & Alegre, 2002). These examples support the idea that tocochromanols are involved in abiotic stress responses of plants. However, the effect of low P-induced stress on tocochromanol concentrations in maize has not been unraveled yet.

2.4.2 Biological function and importance of tocochromanols for human nutrition

One of the most important functions of tocopherols is the ability to act as chain-breaking antioxidant, with α-tocopherol being the most potent peroxyl radical scavenger within this group of compounds (Burton & Ingold, 1981). In this mechanism, a hydrogen atom is transferred for instance from α-tocopherol to a lipid peroxyl radical, thereby, resulting in a stabilized tocopheroxyl radical, which can then be reduced back to α-tocopherol in the presence of ascorbic acid (Doba et al., 1983; Kumar et al., 2020). Thus, α-tocopherol together with other antioxidants counteract lipid peroxidation by limiting the formation of detrimental ROS and lipid peroxyl radicals (Munné-Bosch, 2005). The concentration of α-tocopherol in extracts of dry leaves have positively been

correlated (r = 0.93) with the antioxidant activity, providing evidence that α-tocopherol is the major lipid-soluble antioxidant in leaves (Mallet et al., 1994).

In the absence of ascorbic acid, α-tocopherol is completely oxidized and oxidation products such as α-tocopherylquinone are formed (Liebler et al., 1989). This oxidation mechanism is not only restricted to α-tocopherol. For instance, an increase in γ-tocopherylquinone accompanied by a decrease in γ-tocopherol concentrations was observed during frying of rapeseed oil (Kreps et al., 2017). Tocopherols are required as antioxidants in photoprotection of photosystem II of photosynthetic organisms, by scavenging singlet oxygen and to maintain thylakoid membrane stability (Havaux et al., 2005; Trebst et al., 2002). The latter is also true for tocotrienols as reported for genetically modified tobacco leaves and the authors suggested that tocotrienols serve as antioxidants in seeds (Matringe et al., 2008). Tocopherols also protect polyunsaturated fatty acids against oxidation in seeds of *Arabidopsis*, where they extend the longevity of seeds during storage and reduce ROS formation during seedling development (Sattler et al., 2004). In *Arabidopsis* plants that were genetically modified to have a defective tocopherol synthetic pathway, an increased sensitivity to low temperatures and less leave-to-root photoassimilate transport have been observed, in comparison to wild type plants. This indicates a crucial role of tocopherols in low-temperature adaptations and phloem loading (Maeda et al., 2006). In summary, tocochromanols in plants reveal important functions in cold-resistance, seed storage, germination, and abiotic stresses which are not necessarily limited to their function as antioxidant (Falk & Munné-Bosch, 2010).

In humans, α-tocopherol is considered as essential micronutrient (Galli et al., 2017). It was first described one hundred years ago, in 1922, as an unknown compound present in foods having indispensable functions as a factor for reproduction of rats (Evans & Bishop, 1922). Similar to carotenoids, tocopherols and tocotrienols are obtained from the diet or from food supplements (Lemcke-Norojärvi et al., 2001; Rasool et al., 2008; Wagner et al., 2001). After ingestion, α-tocopherol is captured in mixed micelles together with other lipids and bile salts prior to uptake by the enterocytes (Traber et al., 1990). However, the percentage bioaccessibilities of α-tocopherol varies greatly among food matrices from 0.47% for apples up to 100% in lettuce (Reboul, Richelle, et al., 2006). In addition, infrared heating of maize significantly lowered the

bioaccessibilities of α-, β-, γ-tocopherols and tocotrienols compared to untreated maize (Hossain & Jayadeep, 2021).

In addition to passive processes, the proteins SR-B1 and Niemann-Pick C1-like 1 mediate the uptake of vitamin E incorporated into mixed micelles across the membrane of the enterocyte (Narushima et al., 2008; Reboul et al., 2006). Next, α-tocopherol is either packed into chylomicrons or is associated with high-density lipoprotein and secreted from the basolateral side of the enterocyte into the lymphatic system (Anwar et al., 2006, 2007). During the blood circulation, fats in the chylomicrons are hydrolyzed by lipoprotein lipase (Kiyose et al., 1997). α-Tocopherol, transported by lipoproteins and these chylomicrons remnants, reaches the liver, where the cellular uptake via endocytosis is supported by low-density lipoprotein receptors (Bjørneboe et al., 1987; Herz et al., 1995). Within hepatocytes, *RRR*-α-tocopherol compared to β-, γ-, or δ-tocopherol is preferentially bound by the α-tocopherol transfer protein and re-secreted into the blood circulation by the ATP-binding cassette transporter A1 (Hosomi et al., 1997; Shichiri et al., 2010). Alternatively, tocopherols and tocotrienols are metabolized by cytochrome P450 enzymes starting with ω-hydroxylation of the side-chains, followed by β-oxidations, and finally resulting in short-chain metabolites (Schmölz et al., 2016). The latter mostly undergo enzymatic glucuronidation and are excreted by the urine (Yoshikawa et al., 2005; Zhao et al., 2010).

Similar to plants, α-tocopherol mainly acts in humans as antioxidant and reduces oxidative stress, for example in plasmatic membranes of myocytes (Howard et al., 2011). Due to its essentiality, an acceptable daily intake of α-tocopherol of 11 mg for adult women and 13 mg per day for adult men is proposed by the European Food and Safety Authority (EFSA Panel of Dietetic Products, 2015).

3 Aims of the doctoral studies

The imbalance in the global P-cycle and limited P-resources demand a reduction and more targeted application of phosphate fertilizer in agriculture. Since P is involved in the biosynthesis of partly essential compounds for animal and human nutrition, such as tocopherols, the main hypothesis was that a reduced phosphate-availability in soil affects the concentrations of these organic compounds in grains during cultivation of maize. For study purposes, maize crop was chosen because it requires high amounts of P for optimal plant growth compared to other major crops for food and feed consumption (Müller & Zhang, 2019; Ranum et al., 2014). Furthermore, changes in the chemical composition, especially of phytate, were studied in maize grains, as this may alter oxidation processes in maize-based products during processing and digestion.

To address the main hypothesis, the **first aim** was to investigate the influence of phosphate fertilization on soluble and insoluble (poly)phenols, carotenoids, and tocochromanols in maize (*Zea mays* L.) grains. Concentration in grains of maize plants cultivated with or without phosphate fertilization were compared at a site with low plant-available phosphate in soil. In addition, the impact of sowing time on concentrations of these compounds was analyzed as additional factor (Chapter 2).

In a follow-up study, the influence of phosphate fertilization, location, and maize varieties on fatty acids, carotenoids, and tocochromanols in maize grains was analyzed. Thus, the **second aim** was to identify one-way, two-way, and three-way interactions of the aforementioned parameters on concentrations of fatty acids, carotenoids, and tocochromanols in maize grains. For this purpose, eight commercially available maize hybrids were grown at three sites with or without phosphate fertilization followed by quantitative analyses of those compounds in the maize grains (Chapter 3).

Furthermore, it was considered that elevated phytate concentrations in maize may reduce metal-induced oxidation due the high affinity of phytate to iron and zinc. Since this indirect antioxidant effect of phytate has rarely been investigated, the **third aim** was to elucidate if different phytate concentrations affect the concentrations of carotenoids, tocochromanols, unsaturated fatty acids, and oxidation products (α-tocopherylquinone, malondialdehyde) in maize porridges after heating and three-stage in vitro digestion. In this context, the influence of phytate on the digestive stability, solubility and micellarization efficiency of tocochromanols and carotenoids in maize was studied (Chapter 4).

Chapter 2

(Poly)phenols, carotenoids, and tocochromanols in corn (*Zea mays* L.) kernels as affected by phosphate fertilization and sowing time

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(Poly)phenols, Carotenoids, and Tocochromanols in Corn (*Zea mays* L.) Kernels As Affected by Phosphate Fertilization and Sowing Time

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Supporting Information

ABSTRACT: Corn (*Zea mays* L.) growth and development is often limited by the availability of phosphate. We thus hypothesized that phosphate fertilization may increase the contents of (poly)phenols, carotenoids, and tocochromanols (vitamin E) in corn grains. Corn plants cultivated on a soil fertilized with 44 kg phosphorus/ha were compared to plants grown on soil with low plant-available phosphate (1.6 mg CAL-P/100 g of soil), each sown early (April) and late (May) in a randomized field experiment. HPLC-DAD-(HR)-ESI-MS^n revealed 19 soluble and 10 insoluble (poly)phenols, comprising phenolic acids, phenolic amines, diferulic, and triferulic acids in corn grains. Contents of individual (poly)phenols, carotenoids, and tocochromanols in whole grains were significantly ($p < 0.05$) increased by sowing time, but not by phosphate fertilization. In conclusion, low phosphate availability did not impair the biosynthesis of (poly)phenols, carotenoids, and tocochromanols in corn grains.

KEYWORDS: *phosphate deficiency, polyphenols, vitamin E, carotenoids, Poaceae, maize grain*

INTRODUCTION

Phosphorus (P) is an essential and often limiting plant nutrient. Thus, mineral fertilizers are applied to arable lands to improve crop production.[1] Owing to the growing demand for phosphate fertilizers, the global phosphorus reservoirs are estimated to be exhausted within the next century.[2] Yet, it is assumed that global phosphate fertilization may be decreased by 38% for major cereals including corn without affecting grain yields.[3] However, the influence of a minimized application of phosphate fertilizers on the accumulation of vitamins and phytochemicals in corn has not yet been investigated.

In plant cells, P is an essential constituent of phospholipids, nucleic acids, and phosphorylated metabolic intermediates.[4] For instance, in the early stage of tocochromanol (tocopherol and tocotrienol) biosynthesis in corn, phosphorus is involved as geranylgeranyl and phytyl diphosphate.[5] The carotenoid precursor phytoene is generated by the dimerization of two geranylgeranyl diphosphates.[6] Diversity of carotenoids results from sequential desaturation, isomerization, and cyclization of phytoene. Additional hydroxylations result in xanthophylls, e.g., lutein and zeaxanthin, occurring in sweet corn.[7,8] Carotenoids with at least one β-ring structure have provitamin A activity, thus being important for human nutrition. The latter is also true for tocopherols and tocotrienols (vitamin E), which are health-beneficial[9] and, in the case of α-tocopherol, essential micronutrients.[10]

In a long-term fertilization experiment between 1940 and 1990, crop yields of corn cultivated without P fertilization declined by 50% compared to those supplied with nitrogen, phosphorus, and potassium.[11] In order to optimize P management for crops, not only crop yields but also nutritional quality, including micronutrient and phytochemical compositions, need to be considered.[12] Therefore, the impact of available phosphate and sowing time on the concentrations of (poly)phenols, carotenoids, and vitamin E (tocopherols and tocotrienols) in corn grains was investigated in the present study, which includes compound identification using state-of-the-art analytical instruments such as high-performance liquid chromatography coupled with high-resolution mass spectrometry.

MATERIALS AND METHODS

Chemicals. L(+)-Ascorbic acid (purity ≥99%), butylated hydroxytoluol (BHT), 1,4-dioxane, ethanol, *n*-hexane, potassium hydroxide

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solution (50%, *w*/*v*), glacial acetic acid, *p*-coumaric acid, caffeic acid (both purity ≥95%), and sodium chloride (purity ≥99%) were obtained from Carl-Roth (Karlsruhe, Germany). Acetonitrile and methanol were purchased from J.T. Baker (Giwice, Poland). (all-*E*)-*β*-*Apo*-8′-Carotenal (purity ≥96%), diethyl ether (≥99%, inhibitor-free), ferulic acid, methoxyamine hydrochloride (both purity ≥98%), *α*-carotene, and *α*-, *β*-, *γ*-, *δ*-tocopherol and -tocotrienol standards (purity at least ≥95.5%) were from Sigma-Aldrich (Taufkirchen, Germany). Hydrochloric acid was obtained from VWR (Fontenay-sous-Bois, France). Ethyl acetate and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and formic acid from Th. Geyer (Renningen, Germany). L-Tryptophan (purity ≥99.5%) was from Fluka Chemie (Buchs, Switzerland). Lutein, zeaxanthin, *β*-cryptoxanthin, and *β*-carotene (purity 99% or higher) were obtained from Extrasynthèse (Genay, France). Purified deionized water was prepared by a Milli-Q (Millipore, Billerica, MA, USA) or an arium 611 (Sartorius, Göttingen, Germany) water treatment system. All solvents and standards were of HPLC grade or higher quality.

Plant Material and Sample Preparation. A randomized field experiment with yellow corn (*Zea mays* L.) variety 'Amagrano' (KWS, Einbeck, Germany) was started in 2018 at an experimental station in Freising, Germany (48°23′53.1″N 11°42′35.7″E). In 2018, field conditions were as follows: 740.1 mm annual average precipitation, 10.3 °C annual average temperature, soil pH 6.9 (determined in calcium chloride), and silty-loam as soil type. The crop rotation was corn, winter wheat, and winter barley. Plant-available phosphate content in the top-soil was determined[13] as 1.6 mg CAL-P per 100 g of soil representing a low P level (1.5−3.0 mg CAL-P/100 g soil) according to the Association of German Agricultural Analytic and Research Institutes (VDLUFA).[14] Corn was sown on April 12th (early) and May 3rd (late), 2018. Early- and late-sown samples were grown without phosphate fertilizer as control or with 44 kg P/ha in form of triple superphosphate. For each P fertilization and sowing time, four replicate plots with a plot size of 60 m^2 each and a plant density of 11000 plants per ha were used. Nitrogen fertilizer was applied at a dosage of 200 kg N/ha on May 23rd, 2018. Flowering commenced at the end of June (approximately 478 growing degree days, GDD) for the early-sown corn plants and at the beginning of July (approximately 500 GDD) for the late-sown corn plants. Grains were harvested at full maturity with a plot harvester on September 26th, 2018, corresponding to 1255 and 1171 GDD for the early- and the late-sown corn plants, respectively. The grain yield was recorded gravimetrically in the plot harvester for total fresh matter yield. A subsample was dried at 60 °C for the determination of dry matter yield. Grain samples were dried at 60 °C for 4 days mimicking usual postharvest procedures. The mean dry matter of the kernels was 80%. Kernels from each plot were digested with nitric acid and hydrogen peroxide, and the P concentration in the solution was determined calorimetrically.[15,16]

For the analysis of (poly)phenols, carotenoids, and tocochromanols, whole corn grains were transported to the laboratory in Hohenheim and stored in the dark at −80 °C. Prior to analysis, corn grains from each plot were ground with an electric coffee grinder (VeoHome, Les Étilleux, France). The resulting particle size, examined by a Mastersizer 2000 particle size analyzer (Malvern Panalytical, Malvern, U.K.), had an average Sauter mean diameter of $d[3,2] = 57.9 \pm 0.2$ μm.

Analyses of (Poly)phenols, Carotenoids, and Tocochromanols. *Extraction and HPLC-DAD-(HR)-ESI-MS^n Analyses of (Poly)phenols.* Two (poly)phenolic fractions were extracted from ground corn based on a previously reported method with slight modifications.[17] The first fraction comprised soluble free and conjugated (poly)phenols (termed soluble (poly)phenols in the following sections). The second fraction contained bound (poly)phenolic constituents (insoluble (poly)phenols).[18] Briefly, an aliquot of 100 mg of ground corn kernels was weighed into a centrifuge tube and 3 mL of aqueous methanol (80%, *v*/*v*) was added. The suspension was homogenized for 30 s with an Ultra-Turrax T25 homogenizer (Ultra-Turrax T25 homogenizer, Janke & Kunkel, Staufen, Germany) and centrifuged at 1718*g* for 10 min. The supernatant was collected, and the solid remainder re-extracted twice, each with 2 mL of aqueous methanol (80%, *v*/*v*), as described above. The combined supernatants were evaporated with a rotary evaporator at a pressure of 0 mbar and a temperature of 30 °C (soluble (poly)phenols).

For the insoluble (poly)phenolic fraction, the residual solid matter in the centrifuge tube was resuspended in 2 mL of aqueous NaOH solution (2 mol/L) and stirred for 4 h at a temperature of 20 ± 1 °C. Subsequently, the solution was acidified with HCl (6 mol/L) to pH 2, and 2 mL of purified water was added. Liquid−liquid extraction was performed with 3 mL of ethyl acetate, followed by two additional extractions, each with 2 mL. The ethyl acetate fractions were combined and evaporated (insoluble (poly)phenols). The analytes were redissolved in 300 μL of eluent A/eluent B (50/50, *v*/*v*), membrane-filtered (0.45 μm Acrodisc, Pall, Dreieich, Germany), and transferred into HPLC-vials.

Soluble and insoluble (poly)phenols were analyzed on a HPLC series 1100 (Agilent, Waldbronn, Germany) instrument consisting of a G1315B photodiode array detector, a G1316A column oven (30 °C), a G1312A pump (0.6 mL/min), a G1379A degasser, and a G1313A autosampler (10 μL). Chromatographic separation was achieved with a Kinetex C18 reversed-phase column (150 × 4.6 mm i.d., 2.6 μm particle size, Phenomenex, Aschaffenburg, Germany) equipped with a C18 guard cartridge for 4.6 mm ID columns. Eluent A was H_2O and eluent B was methanol, each containing 1% formic acid (*v*/*v*). Gradient elution was applied: 5% to 70% B (15 min), 70% to 100% B (1 min), isocratic at 100% B (2 min), 100% to 5% B (1 min), and hold at 5% (3 min). Chromatograms were recorded at 280 and 320 nm. For identification of individual (poly)phenols, the HPLC system was connected to an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization (ESI) source operating in positive and negative ion modes at a scan range of *m*/*z* 50 to 800. Nitrogen was used as both the drying gas at a flow rate of 11 L/min and the nebulizing gas at a pressure of 60 psi. The dry temperature was set to 365 °C. Data were processed with Data Analysis edition 3.1 (Bruker Daltonik) and Chemstation (Agilent) software.

High-resolution (HR) mass spectra were recorded using a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) connected with an HPLC series 1290 Infinity (Agilent) instrument. Except for the injection volume (7 μL) and the spectrum scan range (190 to 400 nm), the aforementioned chromatographic parameters were applied. MS settings for both polarities were as follows: scan range, *m*/*z* 100 to 800; spray voltage, 4200 V (positive) and 3500 V (negative); shealth gas flow, 60; auxiliary gas flow, 20; and sample heater temperature, 380 °C. XCalibur version 4.0 (Thermofisher Scientific) was used for data analysis. Peak assignment was based on retention times (t_R), UV absorption maxima (λ_{max}), high-resolution mass spectra, and fragmentation patterns. The identities of ferulic, *p*-coumaric, and caffeic acid were additionally verified using authentic reference standards.

For quantitation, linear calibration curves of ferulic (0.1−100 mg/L) and *p*-coumaric acid (0.1−100 mg/L) were established. Detection and quantitation limits for soluble and insoluble *p*-coumaric acid and ferulic acid were between 0.29 and 1.12 ng on column. Recoveries of *p*-coumaric and ferulic acid between 91 ± 2% and 101 ± 7% from the soluble and 88 ± 5% to 94 ± 6% from the insouble fraction were achieved. Diferulic acids, triferulic acids, *N*,*N*′-di-*p*-coumaroylspermidine, *N*-*p*-coumaroyl-*N*′-feruloylputrescine, and *N*,*N*′-diferuloylputrescine were expressed as *p*-coumaric acid equivalents or ferulic acid equivalents, since authentic standards were not commercially available. Quantitation of (poly)phenols was performed in quadruple for each treatment group. Total soluble (poly)phenols were calculated based on the sum of soluble *p*-coumaric acid, ferulic acids, *N*-*p*-coumaroyl-*N*′-feruloylputrescine, *N*,*N*′-di-*p*-coumaroylspermidine, and *N*,*N*′-diferuloylputrescine. Diferulic acids, triferulic acids, as well as *p*-coumaric acid and ferulic acid in the bound fraction were summarized as total insoluble (poly)phenols.

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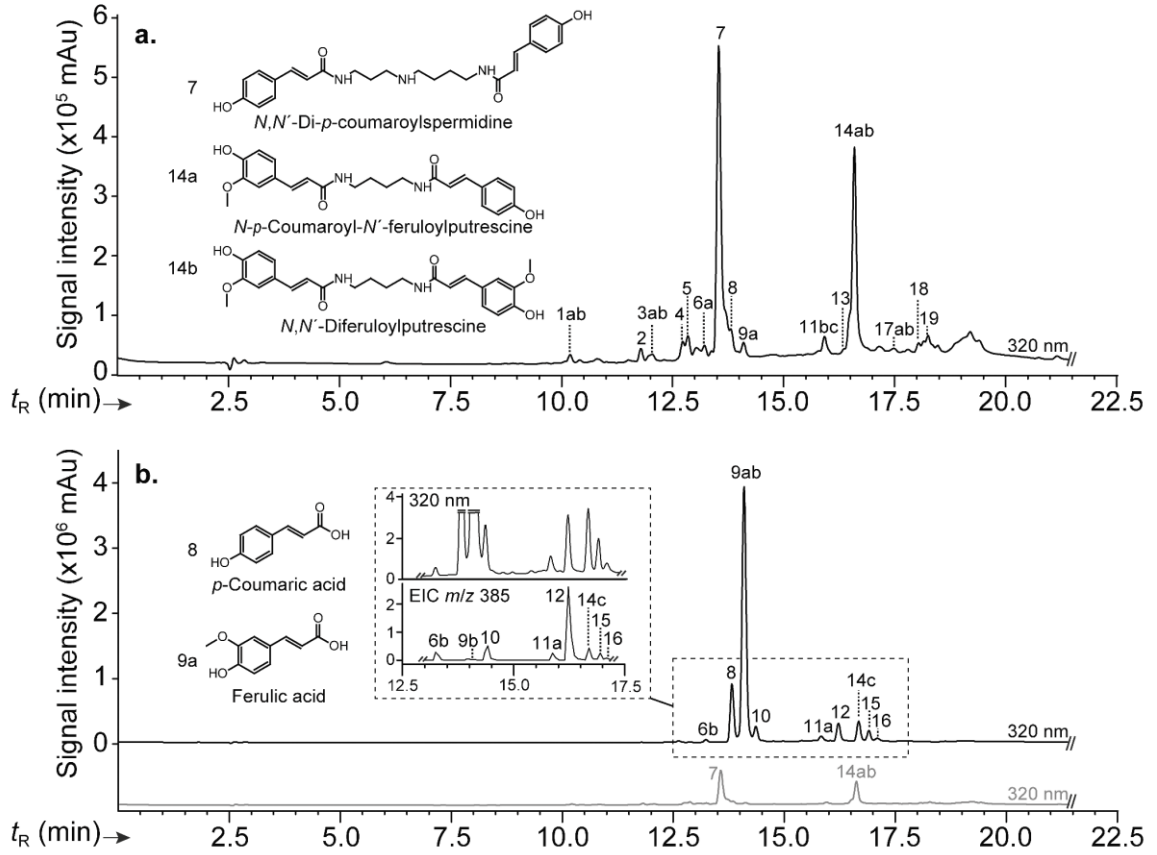

Figure 1. Chromatograms of (a) soluble and (b) insoluble (poly)phenols from a combined (early- and late-sown, with and without P fertilization) corn grain sample recorded at 320 nm. For comparison, the chromatogram of soluble (poly)phenols is displayed in gray scale below the chromatogram of the insoluble fraction. Inlay displays extracted ion current (EIC) chromatogram of m/z 385 in the MS^1 experiment.

Extraction and HPLC-UV/Vis Analyses of Carotenoids. Carotenoid extraction was based on a previously published method with modifications.[19] Experiments about saponification times (0.5, 2, 4 h) and applied temperatures (20, 38, 70 °C) revealed the highest concentrations for carotenoids (β-cryptoxanthin, α- and β-carotene) after saponification at a temperature of 70 °C for 0.5 h (Table S1). Two extraction steps with diethyl ether/hexane (50/50, v/v) yielded recoveries of carotenoids between 90 ± 1% and 97 ± 7% following the optimized extraction method (Tables S2 and S3). In brief, an aliquot of 100 mg of ground corn was mixed with 1 mL of ethanol containing 200 mg/L BHT, 1 mL of ethanol containing 0.12 mL/L β-*apo*-8-carotenal-methyloxime as internal standard (synthesis described elsewhere[19,20]), and 1 mL of aqueous potassium hydroxide solution (50%, w/v). The sample was saponified (0.5 h, 70 °C) in a shaking water bath and subsequently cooled on ice. Two milliliters of aqueous sodium chloride solution (15%, w/v) were added, and the solution neutralized with 1 mL of glacial acetic acid. The solution was extracted with 1 mL of *n*-hexane/diethyl ether (50/50, v/v), vortexed, and centrifuged (3 min, 140g) at 4 °C. The organic layer was transferred into a centrifuge tube, and the aqueous phase was extracted again with 1 mL of the aforementioned extracting agent. The combined supernatants were evaporated to dryness in a vacuum concentrator model RVC 2-33 CDplus (Martin Christ, Osterode am Harz, Germany). The analytes were dissolved in 300 μL of eluent A/eluent B (50/50, v/v) and transferred into an amber HPLC vial.

A volume of 20 μL was analyzed using a Shimadzu (Kyoto, Japan) Prominence HPLC equipped with a LC-20 AT pumping system (1.5 mL/min), a DGU-14A degasser, a CTO-10AS column oven (40 °C), and a SPD-20 A UV/vis detector (450 nm). Separation was achieved with gradient elution and a Develosil RP-Aqueous C30 column (250 × 4.6 mm i.d., 5 μm particle size, Phenomenex, Aschaffenburg, Germany) equipped with a C30 guard column. Eluent A consisted of acetonitrile/methanol (70/30, v/v), and eluent B was acetonitrile/1,4-dioxane/methanol (37/60/3, $v/v/v$). Gradient settings were as follows: 0% to 15% B (5 min), 15% to 100% B (20 min), isocratically hold at 100% B (5 min), 100% to 0% B (2 min), and hold at starting conditions (3 min). For carotenoid quantitation, linear calibration curves of lutein, zeaxanthin (both 0.08 to 6.0 mg/L), β-cryptoxanthin, and α- and β-carotene (each 0.03 to 2.0 mg/L) were used. Detection and quantitation limits for the aforementioned carotenoids were between 0.04 and 0.16 ng on column (Table S3). The total carotenoid content was determined as the sum of the individual pigments.

Extraction and HPLC-FLD Analyses of Tocochromanols. Tocopherols and tocotrienols were extracted and analyzed according to the validated method of Grebenstein and Frank.[21] Briefly, 50 mg of the ground corn sample was suspended in a centrifuge tube with 0.9 mL of H_2O, 0.6 mL of aqueous potassium hydroxide solution (50%, w/v), and 2 mL of ethanol containing 1% ascorbic acid as an antioxidant. Saponification was performed in a shaking water bath at a temperature of 70 °C for 0.5 h. Subsequently, the suspension was cooled on ice. A volume of 25 μL of ethanol containing BHT (1 mg/mL), 1 mL of H_2O, 0.6 mL of glacial acetic acid, and 2 mL of hexane were admixed. After centrifugation (3 min at 140g) at 4 °C, the

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organic phase was collected followed by three additional extraction steps each with 2 mL of *n*-hexane. The combined supernatants were evaporated at 20 °C in a vacuum concentrator (Martin Christ, Osterode am Harz, Germany). Analytes were redissolved in 300 μL of methanol and transferred into amber vials for HPLC analysis.

The Shimadzu HPLC (LC-10AD) consisted of a LC-10AT pumping system, a CTO-10AS column oven (temperature, 40 °C), and a RF-10A fluorescence detector (excitation wavelength, 292 nm; emission wavelength, 325 nm). Separation was achieved using a Kinetex pentafluorophenyl column (100 × 4.6 mm i.d., 2.6 μm particle size, Phenomenex, Aschaffenburg, Germany). Methanol/water (85/15, *v*/*v*) was used as the mobile phase with isocratic elution (20 min) at a flow rate of 1.3 mL/min. The injection volume was 10 μL. Chromatograms were recorded and processed by LabSolutions software version 5.85 (Shimadzu). External calibration curves of tocopherols and tocotrienols were established within the concentration range of 0.03 and 10.54 mg/L with seven calibration points. Total tocochromanols were represented as the sum of the aforementioned tocopherols and tocotrienols.

Detection Limit, Quantitation Limit, Linearity, and Recovery. Recoveries were determined in triplicate by standard addition experiments at low and high spike levels within the calibration range. Detection and quantitation limits were calculated from linear calibration curves based on the slope and the standard deviation of the response according to the guidelines from the International Council for Harmonisation (ICH).[22] Linearity was assessed based on correlation coefficients of the calibration curves.

Statistical Analyses. Levene's test ($p < 0.01$) and Shapiro Wilk's test ($p < 0.05$) were performed to examine normality and homogeneity of variances (Table S4). Concentrations of analyzed compounds in the four groups (early- and late-sown samples, each with (P44) or without phosphate fertilization (P0)) were given as mean ± standard deviation or median with interquartile range (25% and 75% percentile), as appropriate. Differences between groups were determined using one-way ANOVA with the Bonferroni posthoc test (data sets following normal distribution and homogeneity of variances), Welch's test with the Dunnett-T3 posthoc test (normal distributed data sets showing heterogeneous group variances), or the pairwise Kruskal–Wallis test with Bonferroni correction (data sets without normal distribution and showing heterogeneous group variances) and by use of SPSS statistics software version 25 (IBM, Armonk, NY, USA).

For informative purposes, the impact by P fertilization or sowing time, as well as interactions between P fertilization and sowing time on concentrations of (poly)phenols, carotenoids, and tocochromanols, was analyzed using two-way ANOVA and GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). Linear correlations between grain P content and concentration of (poly)-phenols, carotenoids, or tocochromanols were evaluated by Pearson's correlation or Spearman's rank order correlation coefficients. Statistical significance was considered at a *p*-value < 0.05.

■ RESULTS AND DISCUSSION

Total P Concentration and Grain Yield. The total P concentrations of grains were 3.20 or 3.11 mg/g DW in the P-fertilized treatments and 3.02 or 2.91 mg/g DW in the control without P application for the early- or the late-sowing dates, respectively. The P concentrations in the grains were slightly lower than the average P concentration described in corn grains from 28 corn varieties grown in Germany (3.14 ± 0.51 mg/g DW),[23] but above the reported critical value of 2.8 mg P/g dry weight.[24] For the early-sown plants, grain yield was 7.64 t dry weight (DW) per ha in the control without P treatment and 7.71 t DW per ha in the P-fertilized plots. For the late-sowing date, grain yield was 8.12 and 7.87 t DW per ha in the control without and in the treatment with P fertilizer application, respectively. Neither the differences in grain yield nor those in total P concentration were statistically significant ($p < 0.05$) between the treatments.

LC-MS Analyses of (Poly)phenols. The soluble and the insoluble (poly)phenols of the corn grains comprised 19 and 10 compounds, respectively. The corresponding chromatograms and spectrometric data of the two (poly)phenolic fractions are summarized in Figure 1 and Table 1, respectively.

Identification of Soluble (Poly)phenols. Characteristic deprotonated molecules $[M - H]^-$ at *m*/*z* 179.0343, 163.0391, and 193.0500 were revealed for peaks 3a, 8, and 9a assigned to caffeic, *p*-coumaric, and ferulic acid, respectively (Table 1). In agreement with the literature, MS^2 fragment ions resulting from decarboxylations $[M - H - 44]^-$ were detected at *m*/*z* 135, 119, and 149. Ferulic acid additionally displayed fragment ions at *m*/*z* 178 ($[M - H - 15]^-$) and 134 ($[M - H - 44 - 15]^-$) from the radical loss of CH_3 (15 amu) from the methoxy group.[25] The identities were substantiated by comparing t_R, UV absorption, and mass spectra results to those of authentic reference standards. One aromatic amino acid (1a) was identified as L-tryptophan in the soluble (poly)phenolic fraction and was confirmed by an authentic standard.

In addition to phenolic acids, phenolic amines, mainly deriving from *p*-coumaric and ferulic acid (1b, 3b, 4, 5, 7, 11bc, 14ab, 18, 19) were present in the soluble (poly)phenolic fraction. Figure 2 exemplarily illustrates the ESI(−)- and ESI(+)-MS^2 spectra of compound 14a assigned to *N*-*p*-coumaroyl-*N*′-feruloylputrescine. The ESI(−)-MS^2 experiment of the deprotonated molecules $[M - H]^-$ at *m*/*z* 409.1770 displayed abundant fragment ions at *m*/*z* 289 and 259 resulting from the elimination of 4-vinyl-phenol (120 amu) and 2-methoxy-4-vinylphenol (150 amu), respectively. The loss of 4-vinyl-phenol and CH_3 yielded *m*/*z* 274 ($[M - H - 120 - 15]^-$).

In the positive ion mode, compound 14a displayed protonated molecules $[M + H]^+$ at *m*/*z* 411.1914. The eliminations of 120 and 150 amu described above resulted in fragment ions at *m*/*z* 291 and 261. In addition, the most abundant signals at *m*/*z* 265 and 235 were generated by the loss of the *p*-coumaroyl (146 amu) and feruloyl (176 amu) moieties, respectively. Subsequent elimination of ammonia (17 amu), possibly generated by the cyclization of the putrescine residue, resulted in fragment ions at *m*/*z* 248 ($[M + H - 146 - NH_3]^+$) and 218 ($[M + H - 176 - NH_3]^+$). Ammonia elimination was also observed from the protonated molecule (*m*/*z* 394). Likewise, the amide bond cleavage with subsequent ammonia elimination has been described for *N*,*N*′,*N*″-tris-hydroxycinnamoyl spermidine.[26] The fragment ions at *m*/*z* 177 and 145 confirmed a feruloyl and at *m*/*z* 147 a *p*-coumaroyl conjugate (see proposed structures in Figure 2c). The occurrence of *p*-coumaroyl-feruloylputrescine has been previously reported in corn bran and fiber, and its electron impact mass spectrum resembled the ESI(+)-MS^2 spectrum.[27] *N*,*N*′-di-*p*-Coumaroylputrescine (13) and *N*,*N*′-diferuloylputrescines (11c, 14b) followed the fragmentation pattern described above. Both phenolic amines were isolated before from corn bran.[28] Further phenolamides comprised *N*-*p*-coumaroyl and *N*-feruloyltryptamine (18, 19). The ESI(+)-MS^2 spectra of the $[M + H]^+$ precursors at *m*/*z* 307.1439 and 337.1546 displayed base peak fragment ions at *m*/*z* 147 and 177 ($[M + H - 160]^+$), respectively, resulting from the elimination of tryptamine (160 amu).

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Table 1. HPLC-DAD-(HR)-ESI-MS Data of Soluble and Insoluble (Poly)phenols from Corn Treated with Different Doses of Phosphate Fertilizer and Sown at Two Different Dates[a,b]

no.	t_R (min)	λ_{max} (nm)	$[M - H]^-$ (*m/z*)	ESI(−)-MS^n experiment (*m/z*, % base peak intensity)	$[M + H]^+$ (*m/z*)	ESI(+)-MS^n experiment (*m/z*, % base peak intensity)	proposed structure	fraction
1ab	10.2	sh271, 279, 288	203.0821 (203.0826)	[203]: 159 (100), 142 (26), 116 (64)	205.0971 (205.0972)	[205]: 188 (100), 146 (2)	L-tryptophan[c]	SP
			n.d.	n.d.	265.1547 (265.1547)	[265]: 248 (35), 177 (100), 145 (37), 114 (23) [265→177]: 145 (100)	N-feruloylputrescine	SP
2	11.8	sh300, 324	253.0718 (253.0718)	[253]: 179 (12), 161 (16), 135 (100)	n.d.	n.d.	caffeoylglycerol	SP
3a	12.0	sh298, 324	179.0343 (179.0350)	[179]: 135 (100)	n.d.	n.d.	caffeic acid[c]	SP
3b	12.1	n.d.	468.2141 (468.2140)	[468]: 332 (100), 306 (26), 161 (14), 135 (4) [452→332]: 332 (100)	470.2285 (470.2286)	[470]: 453 (56), 308 (93), 291 (18), 220 (100), 163 (31) [470→220]: 220 (100), 163 (21), 145 (7)	N,N′-dicaffeoylspermidine	SP
4	12.7	sh297, 314	452.2194 (452.2191)	[452]: 332 (7), 316 (65), 306 (100), 161 (3), 145 (13), 135 (65) [452→306]: 306 (100)	454.2337 (454.2336)	[454]: 437 (59), 308 (22), 292 (49), 275 (6), 220 (100), 204 (7), 163 (35), 147 (10) [454→220]: 220 (100), 204 (13), 163 (28), 147 (13)	N-caffeoyl-N′-*p*-coumaroylspermidine (1)	SP
5	12.8	297, 307	452.2193 (452.2191)	[452]: 332 (100), 316 (30), 306 (48), 161 (2), 145 (9), 135 (28) [452→332]: 332 (100)	454.2336 (454.2336)	[454]: 437 (73), 308 (22), 292 (51), 275 (13), 220 (33), 204 (100), 163 (35), 147 (28) [454→204]: 147 (100)	N-caffeoyl-N′-*p*-coumaroylspermidine (2)	SP
6a	13.2	sh299, 310	237.0766 (237.0769)	[237]: 163 (43), 145 (22), 119 (100)	n.d.	n.d.	*p*-coumaroylglycerol	SP
6b	13.2	337	385.0929 (385.0929)	[385]: 341 (100) [385→341]: 341 (100), 326 (11), 297 (7), 282 (5)	341.1019[e] (341.1020) 409.0893[d] (409.0894)	[341]:[c] 323 (100), 297 (92), 265 (66), 237 (4)	8,8′-DiFA, aryltetralin form (diferulic acid 1)	IP
7	13.5	299, 308	436.2243 (436.2242)	[436]: 316 (100), 290 (8), 145 (13) [436→316]: 316 (100), 273 (8), 145 (1)	438.2388 (438.2387)	[438]: 421 (43), 292 (33), 274 (11), 204 (100), 147 (74)	N,N′-di-*p*-coumaroylspermidine	SP
8	13.8	sh299, 310	163.0391 (163.0401)	[163]: 119 (100)	165.0546 (165.0546)	n.d.	*p*-coumaric acid[c]	SP, IP
9a	14.1	sh293, 325	193.0500 (193.0506)	[193]: 178 (91), 149 (100), 134 (16)	195.0652 (195.0652)	[195]: 177 (100) [195→177]: 145 (100)	ferulic acid[c]	SP, IP
9b	14.1	n.d.	385.0929 (385.0929)	[385]: 341 (56), 326 (7), 297 (64), 282 (100), 281 (30), 173 (19), 159 (41), 145 (3), 123 (2)	387.1070 (387.1074)	[387]: 369 (100), 351 (76), 343 (48), 341 (8), 325 (54), 307 (21), 297 (4), 293 (13), 265 (4), 263 (68), 245 (18), 219 (37), 201 (28), 193 (19), 173 (10)	8,8′-DiFA (diferulic acid 2)	IP
10	14.3	320	385.0931 (385.0929)	[385]: 341 (100), 326 (7), 297 (86), 282 (23), 267 (4) [385→341]: 341 (100), 326 (3), 297 (13)	387.1073 (387.1074)	[387]: 369 (100), 341 (12), 337 (1), 309 (58)	8,5′-DiFA (diferulic acid 3)	IP
11a	15.8	sh300, 322	577.1351 (577.1352)	[577]: 533 (73), 489 (100), 461 (62), 445 (25), 341 (73), 326 (8), 297 (15), 282 (8), 267 (4), 163 (2)	n.d.	n.d.	triferulic acid (1)	IP
11bc	15.9	294, 313	409.1770 (409.1770)	[409]: 289 (100), 274 (8), 259 (64), 149 (15), 135 (72), 119 (21) [409→289]: 289 (100), 274 (37), 149 (25), 134 (9)	411.1914 (411.1914)	[411]: 394 (4), 291 (21),265 (100), 261 (17), 248 (12), 235 (87), 218 (23), 177 (50), 147 (49), 145 (13) [411→265]: 248 (100), 206 (40), 177 (29), 145 (3)	N-*p*-coumaroyl-N′-feruloylputrescine (1)	SP
			439.1875 (439.1875)	[439]: 289 (100), 274 (15), 149 (14), 135 (26) [439→289]: 289 (100), 274 (31), 149 (53), 134 (9)	441.2021 (441.2020)	[441]: 291 (8), 265 (92), 248 (28), 177 (100), 145 (63) [441→265]: 248 (65), 206 (28), 177 (100), 145 (11)	N,N′-diferuloylputrescine (1)	SP
12	16.2	325	385.0929 (385.0929)	[385]: 385 (12), 370 (9), 341 (100), 326 (26), 282 (38) [385→341]: 341 (100), 326 (2), 282 (2)	387.1071 (387.1074)	[387]: 369 (12), 351 (18), 325 (100), 323 (2), 307 (27), 293 (17), 283 (12)	5,5′-DiFA (diferulic acid 4)	IP
13	16.4	n.d.	379.1667 (379.1663)	[379]: 259 (100), 119 (14) [379→259]: 145 (13), 119 (100)	381.1808 (381.1809)	[381]: 364 (1), 261 (27), 235 (100), 218 (29), 147 (36)	N,N′-di-*p*-coumaroylputrescine	SP
14ab	16.6	sh295, 319	409.1770 (409.1769)	[409]: 289 (100), 274 (12), 259 (55), 149 (8), 135 (64), 119 (13) [409→289]: 289 (100), 274 (38), 149 (88), 134 (12)	411.1914 (411.1914)	[411]: 394 (5), 291 (17), 265 (100), 261 (30), 248 (19), 235 (84), 218 (26), 177 (55), 147 (52), 145 (14) [411→265]: 248 (100)	N-*p*-coumaroyl-N′-feruloylputrescine (2)	SP
			439.1875 (439.1875)	[439]: 289 (100), 274 (13), 149 (13), 135 (34) [439→289]: 289 (100), 274 (22), 149 (45), 134 (13)	441.2020 (441.2020)	[441]: 291 (12), 265 (100), 248 (4), 177 (69), 145 (7) [441→265]: 248 (100), 206 (28), 177 (88), 145 (3)	N,N′-diferuloylputrescine (2)	SP

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Table 1. continued

no.	t_R (min)	λ_{max} (nm)	$[M - H]^-$ (m/z)	ESI(−)-MS^n experiment (m/z, % base peak intensity)	$[M + H]^+$ (m/z)	ESI(+)-MS^n experiment (m/z, % base peak intensity)	proposed structure	fraction
14c	16.7	sh293, 327	385.0929 (385.0929)	[385]: 341 (43), 326 (9), 313 (100), 298 (16), 282 (10), 193 (65), 179 (10), 149 (5), 134 (7) [385→313]: 313 (100), 298 (53), 283 (4), 269 (2), 252 (2)	387.1073 (387.1074)	[387]: 369 (100), 351 (88), 325 (31), 263 (36), 219 (32), 204 (4), 201 (5), 193 (23), 177 (4), 149 (2)	8-*O*-4′-DiFA (diferulic acid 5)	IP
15	16.9	324	385.0929 (385.0929)	[385]: 341 (100), 326 (5), 297 (3), 282 (3) [385→341]: 341 (100), 326 (3), 297 (6), 282 (2)	387.1073 (387.1074)	[387]: 369 (36), 351, (65), 343 (100), 341 (4), 325 (81), 307 (35), 297 (3), 293 (12), 265 (2), 263 (29), 245 (36), 219 (7), 201 (7)	8,5′-DiFA benzofuran form (diferulic acid 6)	IP
16	17.2	sh299, 320	577.1354 (577.1352)	533 (100), 489 (16), 461 (3), 385 (11), 355 (18), 311 (23), 193 (2)	n.d.	n.d.	triferulic acid (2)	IP
17ab	17.5	289, 318	399.1086 (399.1085)	[399]: 253 (100), 235 (26), 179 (5), 163 (24), 161 (12), 145 (19), 135 (14), 119 (10) [399→253]: 179 (12), 161 (35), 135 (100)	n.d.	n.d.	*p*-coumaroyl-caffeoylglycerol	SP
			429.1192 (429.1191)	[429]: 253 (100), 249 (5), 235 (62), 193 (54), 179 (2), 175 (22), 161 (46), 145 (1), 135 (18) [429→253]: 179 (7), 161 (24), 135 (100	n.d.	n.d.	feruloyl-caffeoylglycerol	SP
18	18.0	291, 315	305.1297 (305.1296)	[305]: 305 (100), 145 (9), 119 (26)	307.1439 (307.1441)	[307]: 290 (12), 147 (100)	*N*-*p*-coumaroyltryptamine	SP
19	19.0	291, 321	335.1403 (335.1401)	[335]: 335 (100), 320 (3), 178 (1), 175 (3), 149 (5), 134 (3)	337.1546 (337.1547)	[337]: 320 (6), 177 (100), 145 (9) [337→177]: 145 (100)	*N*-feruloyltryptamine	SP

[a] t_R, retention time; λ_{max}, UV absorption maxima; sh, shoulder; SP, soluble (poly)phenols; IP, insoluble (poly)phenols; n.d., not detected. [b]Calculated values for the HR-ESI-MS experiments are given in parentheses. [c]In-source fragmentation $[M + H - H_2O - CO]^+$. [d]Sodium adduct $[M + Na]^+$. [e]Verified using an authentic reference standard.

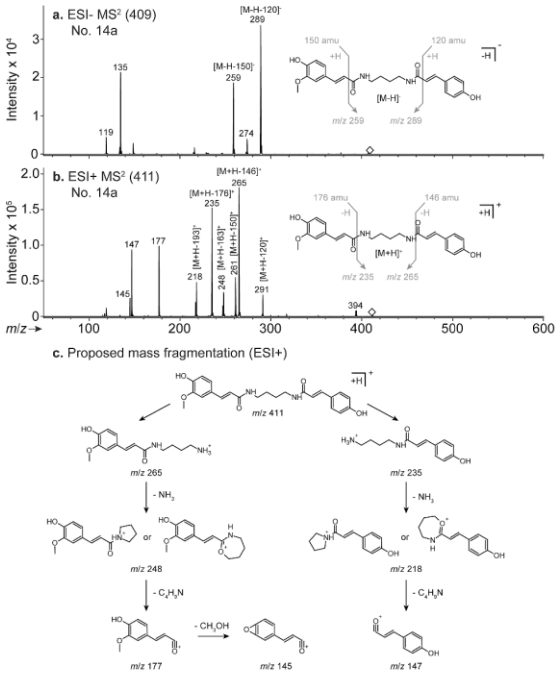

Figure 2. ESI-MS^2 spectra of compound 14a assigned to *N*-*p*-coumaroyl-*N*′-feruloylputrescine in the (a) ESI negative and (b) ESI positive ion mode. The proposed mass fragmentation pattern of compound 14a in ESI positive mode (c) was based on mass spectra of polyamines published previously.[26,50]

The soluble (poly)phenolic fraction additionally comprised the mono- and dihydroxycinnamoyl glycerides caffeoylglycerol (2), *p*-coumaroylglycerol (6a), *p*-coumaroyl-caffeoylglycerol (17a), and feruloyl-caffeoylglycerol (17b). The aforementioned compounds displayed deprotonated molecules $[M - H]^-$ at m/z 253.0718, 237.0766, 399.1086, and 429.1192. Their ESI(−)-MS^2 fragment ions were consistent with previous reports.[29,30] The assignments of caffeoyl- and *p*-coumaroylglycerol were corroborated by their characteristic UV absorption maxima λ_{max} resembling those of the phenolic acid moieties (3a and 8).

Identification of Insoluble (Poly)phenols. The insoluble (poly)phenols comprised *p*-coumaric (8) and ferulic acid (9a), in addition to six less abundant diferulic (6b, 9b, 10, 12, 14c, 15) and two triferulic acids (11a, 16). The diferulic acids displayed deprotonated molecules $[M - H]^-$ between m/z 385.0929 and 385.0931 in the negative ion mode (calculated $[M - H]^-$ at m/z 385.0929). Noteworthily, this precursor was also observed as an in-source fragment ion of triferulic acids (Figure 1b). Despite this occasion, individual diferulic acids were tentatively assigned based on their specific MS^n fragmentations, UV spectra, and elution orders.[17,31−33]

In the ESI(+)-MS^1 spectrum, diferulic acids 2 (9b), 3 (10), 4 (12), and 5 (14c) displayed protonated molecules $[M + H]^+$ between m/z 387.1070 to 387.1073 (calculated $[M + H]^+$ at m/z 387.1074). However, diferulic acid 1 (6b), displayed an abundant sodium adduct $[M + Na]^+$ at m/z 409.0893. In addition, an in-source fragment at m/z 341.1019 ($[M + H - H_2O - CO]^+$) was detected, resulting from the eliminations of water (18 amu) and carbon monoxide (28 amu). Collision-

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Table 2. Quantitation of Soluble (Poly)phenols Extracted from Corn Grown with and without P Fertilization (0 kg/ha, 44 kg P/ha) and Sown at Two Different Dates (Δt = 21 days; early or late)

		concentration [μg/g DW]					*p*-value (two-way ANOVA)		
peak no.	proposed structure	P0 (early)	P44 (early)	P0 (late)	P44 (late)	*p*-value	phosphate fertilization	sowing time	phosphate fertilization × sowing time
7	*N,N′*-di-*p*-coumaroylspermidine[b]	96.8 (85.7−98.3) a	107.7 (88.5−124.9) a	115.3 (74.6−120.4) a	92.5 (72.2−107.6) a	0.477[d]	0.972	0.749	0.210
8	*p*-coumaric acid	24.2 ± 1.4 a	26.5 ± 4.0 a	25.2 ± 5.0 a	22.5 ± 2.0 a	0.434[e]	0.916	0.388	0.170
9a	ferulic acid	13.1 ± 0.6 a	12.1 ± 0.6 a	12.6 ± 1.1 a	12.1 ± 0.6 a	0.288[e]	0.091	0.555	0.498
14ab	*N*-*p*-coumaroyl-*N′*-feruloylputrescine (2), *N,N′*-diferuloylputrescine (2)[c]	72.8 ± 20.6 a	72.6 ± 7.1 a	33.1 ± 1.8 b	34.3 ± 1.4 b	<0.001[e]	0.927	<0.001	0.901
	total soluble (poly) phenols	203.6 ± 25.5 ab	218.2 ± 19.3 a	174.3 ± 35.6 ab	159.7 ± 21.7 b	0.031[e]	0.288	0.006	0.998

[a]DW, dry weight; P0, cultivated without P fertilizer; P44, fertilized with 44 kg P/ha. Early sowing time: April 12, 2018. Late sowing time: May 3, 2018. Concentrations given as mean ± standard deviation (n = 4) or median (interquartile range). Concentrations not sharing a common letter (a, b) represent significant differences between groups ($p < 0.05$). [b]Concentration determined as *p*-coumaric acid equivalents. [c]Concentration determined as ferulic acid equivalents. [d]*p*-value: pairwise Kruskal−Wallis test. [e]*p*-value: one-way ANOVA.

Table 3. Quantitation of Insoluble (Poly)phenols Extracted from Corn Grown with and without P Fertilization (0 kg/ha, 44 kg P/ha) and Sown at Two Different Dates (Δt = 21 days; early or late)

		concentration [μg/g DW]					*p*-value (two-way ANOVA)		
peak no.	proposed structure	P0 (early)	P44 (early)	P0 (late)	P44 (late)	*p*-value (one-way ANOVA)	phosphate fertilization	sowing time	phosphate fertilization × sowing time
6b	8,8′-DiFA, aryltetralin form[b]	15.8 ± 5.6 a	12.1 ± 0.7 a	10.8 ± 0.8 a	11.8 ± 1.9 a	0.141	0.379	0.098	0.137
8	*p*-coumaric acid	286.3 ± 54.2 a	237.5 ± 25.8 ab	201.3 ± 43.4 b	209.0 ± 14.9 ab	0.031	0.299	0.011	0.161
9ab	ferulic acid, 8,8′-DiFA[b]	2252.8 ± 219.4 a	2109.3 ± 120.9 a	1936.6 ± 150.4 a	2098.5 ± 177.0 a	0.130	0.916	0.080	0.099
10	8,5′-DiFA[b]	106.7 ± 15.1 a	88.8 ± 4.9 a	81.1 ± 9.0 a	84.4 ± 18.6 a	0.069	0.283	0.040	0.130
11a	triferulic acid (1)[b]	39.6 ± 13.4 a	30.1 ± 4.8 a	26.1 ± 5.7 a	31.4 ± 9.6 a	0.246	0.644	0.202	0.127
12	5,5′-DiFA[b]	197.1 ± 39.6 a	159.8 ± 13.2 a	151.0 ± 21.7 a	163.3 ± 18.4 a	0.104	0.343	0.117	0.073
14c	8-*O*-4′-DiFA[b]	216.8 ± 47.8 a	181.0 ± 12.1 a	158.1 ± 20.7 a	171.4 ± 16.6 a	0.061	0.438	0.031	0.105
15	8,5′-DiFA benzofuran form[b]	113.4 ± 30.2 a	93.3 ± 5.3 a	87.1 ± 11.1 a	94.0 ± 10.9 a	0.207	0.456	0.161	0.142
16	triferulic acid (2)[b]	89.0 ± 40.9 a	65.7 ± 7.4 a	58.2 ± 7.0 a	58.93 ± 6.6 a	0.195	0.309	0.104	0.281
	total insoluble (poly) phenols	3317.5 ± 409.8 a	2977.5 ± 165.0 a	2710.2 ± 256.1 a	2922.6 ± 261.8 a	0.068	0.220	0.040	0.078

[a]DiFA, diferulic acid; DW, dry weight; P0, cultivated without P fertilizer; P44, fertilized with 44 kg P/ha. Early sowing time: April 12, 2018. Late sowing time: May 3, 2018. Concentrations given as mean ± standard deviation (n = 4). Concentrations not sharing a common letter (a, b) represent significant differences between groups ($p < 0.05$). [b]Concentration determined as ferulic acid equivalents.

induced dissociation of the m/z 341 precursor yielded fragment ions at m/z 323, 297, and 265, possibly resulting from further eliminations of water (18 amu), carbon dioxide (44 amu), as well as carbon dioxide and methanol (32 amu). Besides the described fragment ions, the distinct absorption maxima λ_{max} at 337 nm consolidated the identity of 8,8′-diferulic acid (aryltetralin form).[33] The remaining diferulic acids assigned to 8,8′-diferulic acid (9b) and 8,5′-diferulic acid (10) displayed characteristic fragment ions at m/z 245 ($[M + H - H_2O - C_7H_8O_2]^+$) and m/z 309 ($[M + H - H_2O - CO - CH_3OH]^+$), respectively.[32] The diferulic acid isomers 4 to 6 were tentatively assigned to 5,5′-diferulic acid (12), 8-*O*-4′-diferulic acid (14c), and 8,5′-diferulic acid (benzofuran form, 15), following the elution order on a C18 stationary phase.[31]

In ESI negative mode, compounds 11a and 16 assigned to isomeric triferulic acids displayed deprotonated molecules $[M - H]^-$ at m/z 577.1354 (calculated $[M - H]^-$ at m/z 577.1352). MS^2 fragment ions at m/z 533, 489, 445, 311, 341, 355, 385, 163, and 193 of both compounds matched those previously described for triferulic acids.[34,35] However, an assignment of specific isomers was not possible in our study.

Diferulic and triferulic acids have been previously identified in corn bran[36] and in the outer layer of wheat grain.[37] Here, they are cross-linkers of polysaccharides in the cell wall and contribute to its textural stability.[38] The bioavailability of diferulic acids of mammals has recently been shown in a rat model.[39]

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Quantitation of Soluble and Insoluble (Poly)phenols. All corn samples assessed displayed the above-described qualitative profile of soluble and insoluble (poly)phenols. Abundant peaks of the soluble (Table 2) and the insoluble (poly)phenolic fraction (Table 3) were quantitated by HPLC-DAD to examine possible effects of phosphate fertilization and/or sowing time on the concentrations of (poly)phenols in corn grains. Noteworthily, the total insoluble (poly)phenols in corn kernels (2710.2 to 3317.5 μg/g DW) exceeded those of the soluble fraction (159.7 to 218.2 μg/g DW), thus being consistent with previous observations in corn, wheat, oats, and rice grains.[40] Within the early- and late-sown corn samples, there were no differences in the contents of phenolic acids, diferulic acids, and triferulic acids in the corn grains cultivated under low phosphate conditions compared to those cultivated with phosphate fertilization. Correlations between grain P content and the contents of soluble and insoluble (poly)-phenols were also insignificant. In comparison to corn plants grown under salt stress, an increase in the diferulic acid concentrations of up to 67% has been reported in the shoot elongating zone.[31] According to literature, an accumulation of insoluble (poly)phenols was also observed in corn leaves cultivated under drought stress.[41] Insoluble *p*-coumaric acid contents were significantly higher in early-sown corn samples (286.3 ± 54.2 μg/g of DW) when compared to late-sown samples (201.3 ± 43.4 μg/g of DW) cultivated without phosphate fertilization. Even higher concentrations between 1936.6 to 2252.8 μg ferulic acid equivalents/g of DW were observed for insoluble ferulic acid (9a). The latter was not baseline resolved from 8,8′-diferulic acid (9b) in the insoluble fraction; however, this trace constituent may be neglected (see low abundant EIC signal of *m*/*z* 385 in Figure 1b). In addition to insoluble *p*-coumaric acid, 8,5′- and 8-*O*-4′-diferulic acid were significantly affected by sowing time, when analyzed by two-way ANOVA. The temperature differences during grain filling stage may be responsible for the different contents of insoluble (poly)phenols, especially in dryer and warmer years.[42]

The concentrations of soluble *p*-coumaric acid in all grain samples ranged from 22.5 to 26.5 μg/g of DW and those of soluble ferulic acid from 12.1 and 13.1 μg/g of DW, but they were insignificantly influenced by both sowing time and phosphate fertilization. In a field experiment with soy beans cultivated with 0, 60, 120, and 240 kg/ha of phosphate fertilizer, it was shown that an increase in the application of phosphate fertilizer did not enhance the nutritional quality, including total phenolic contents.[12] When looking at other compartments, such as roots, the concentration of ferulic acid in *Arabidopsis* seedlings increased 2-fold under P-limiting conditions in a hydroponic system.[43] In our study, grain samples sown on the same date but treated with different phosphate doses contained almost identical concentrations of *N*-*p*-coumaroyl-*N*′-feruloylputrescine (14a) and *N*,*N*′-diferuloylputrescine (14b), amounting to 72.6−72.8 and 33.1−34.3 μg ferulic acid equivalents/g of DW for the early- and late-sown samples, respectively. The concentrations of these phenolic amides significantly varied by a factor of two between early- and late-sown samples. A reason for the accumulation of phenolamides in the early-sown samples may be associated with biotic or abiotic stressors as observed for tobacco after attack from herbivorous insects.[44]

Quantitation of Carotenoids, Tocopherols, and Tocotrienols. The influence of phosphate fertilization and/or sowing time on the concentrations of carotenoids and vitamin E derivatives (tocopherols, tocotrienols) were investigated.

Carotenoids. In all corn grain extracts, both xanthophylls (lutein, zeaxanthin, β-cryptoxanthin) and carotenes (α- and β-carotene) were identified (Figure 3), in agreement with published data.[38] The total carotenoid content was between 21.7 ± 1.7 and 25.4 ± 1.0 μg/g of DW. Within the early- and late-sown samples, phosphate fertilization had no effect on total carotenoids and individual provitamin A carotenoids (β-cryptoxanthin, α- and β-carotene; Table 4). For instance, the concentrations of β-carotene were 1.5 ± 0.2 and 1.4 ± 0.2 μg/g of DW in the early-sown samples with and without phosphate application, respectively. This also applied for lutein (11.4 ± 0.9 and 11.0 ± 1.3 μg/g of DW) and zeaxanthin (7.6 ± 0.4 and 7.8 ± 0.4 μg/g of DW). Elevated concentrations of lutein and zeaxanthin in yellow-colored corn grains compared to provitamin A carotenoids was in agreement with those in the literature.[8] Phosphate fertilization had no effect on lutein (p = 0.052) or zeaxanthin concentrations (p = 0.948) in corn grains. In contrast, nitrogen supplementation has been reported to result in increased concentrations of the aforementioned xanthophylls.[45] Nevertheless, within the group of carotenoids, a significant negative correlation between grain P content was observed for lutein (Pearson correlation coefficient, −0.604; p = 0.024), but was not further characterized. No interactions of phosphate fertilization and sowing time were found considering the individual and total carotenoids (Table 4).

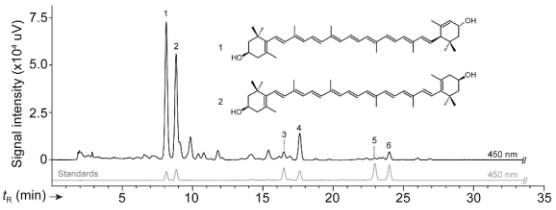

Figure 3. Chromatogram of carotenoids in combined corn grain extracts (early- and late-sown samples with and without P fertilization) recorded at 450 nm. 1: Lutein. 2: Zeaxanthin. 3: β-Cryptoxanthin. 4: β-*apo*-8′-Carotenal-methyloxime (internal standard). 5: α-Carotene. 6: β-Carotene.

Interestingly, diminished concentrations of all carotenoids were observed in the early- compared to the late-sown corn samples. With the exception of α-carotene, all differences were statistically significant (two-way ANOVA, p < 0.05). For instance, the total carotenoid content from corn with phosphate treatment was significantly lower in the early-sown samples with 21.7 ± 1.7 μg/g DW in comparison to the late-sown samples with 24.1 ± 2.0 μg/g of DW. Significant effects of the sowing time on the concentrations of lutein and β-cryptoxanthin in a previous study have been attributed to lower average temperatures during ripening of the early-sown samples.[42] Genotype-dependent effects on the contents of carotenoids, for instance for β-carotene and β-cryptoxanthin in provitamin A-biofortified corn hybrids, should be considered when comparing the results with further studies.[46]

Tocochromanols. The concentrations of γ-tocopherol (11.0 to 13.5 μg/g of DW) and δ-tocopherol (2.5 to 2.9 μg/g of DW) were within the expected concentration range of

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Table 4. Quantitation of Carotenoids, Tocopherols, and Tocotrienols from Corn Grown with and without P Fertilization (0 kg/ha, 44 kg P/ha) and Sown at Two Different Dates (Δt = 21 days; early or late)

	concentration [μg/g DW]					*p*-value (two-way ANOVA)		
compound	P0 (early)	P44 (early)	P0 (late)	P44 (late)	*p*-value	phosphate fertilization	sowing time	phosphate fertilization × sowing time
lutein	11.4 ± 0.9 ab	11.0 ± 1.3 b	12.4 ± 0.9 a	11.5 ± 1.2 ab	0.022[c]	0.052	0.018	0.420
zeaxanthin	7.6 ± 0.4 c	7.8 ± 0.4 bc	9.2 ± 1.0 a	9.0 ± 1.4 ab	<0.001[d]	0.948	<0.001	0.372
β-cryptoxanthin	0.9 ± 0.2 b	1.0 ± 0.2 b	1.5 ± 0.3 a	1.4 ± 0.2 a	<0.001[c]	0.5947	<0.001	0.284
α-carotene	0.4 ± 0.1 a	0.4 ± 0.1 a	0.5 ± 0.1 a	0.4 ± 0.1 a	0.347[c]	0.180	0.223	0.938
β-carotene	1.5 ± 0.2 b	1.4 ± 0.2 b	1.8 ± 0.2 a	1.7 ± 0.2 a	<0.001[c]	0.211	<0.001	0.825
δ-tocotrienol	1.4 (1.3–1.6) b	1.5 (1.4–1.5) ab	1.4 (1.4–1.8) ab	1.7 (1.6–1.8) a	0.016[b]	0.180	0.035	0.297
γ-tocotrienol	3.6 (3.4–4.5) a	3.8 (3.7–5.2) a	3.7 (3.6–4.3) a	4.4 (3.8–4.8) a	0.184[b]	0.058	0.966	0.804
δ-tocopherol	2.9 (2.7–3.9) a	2.5 (2.5–3.4) a	2.5 (2.5–3.4) a	2.8 (2.8–2.8) a	0.054[b]	0.531	0.571	0.128
γ-tocopherol	13.5 ± 0.8 a	12.8 ± 1.3 a	11.0 ± 1.6 b	12.5 ± 2.3 ab	0.004[c]	0.365	0.004	0.024
total carotenoids	21.8 ± 1.3 b	21.7 ± 1.7 b	25.4 ± 1.0 a	24.1 ± 2.0 a	<0.001[c]	0.138	<0.001	0.204
total tocochromanols	22.1 ± 1.9 a	21.4 ± 2.5 ab	19.2 ± 1.5 b	21.5 ± 2.5 ab	0.012[c]	0.2201	0.029	0.022

[a]DW, dry weight; P0, cultivated without P fertilizer; P44, fertilized with 44 kg P/ha. Early sowing time: April 12, 2018. Late sowing time: May 3, 2018. Concentrations given as mean ± standard deviation (n = 12) or median (interquartile range). Concentrations not sharing a common letter (a, b) represent significant differences between groups ($p < 0.05$). [b]*p*-value: pairwise Kruskal–Wallis test. [c]*p*-value: one-way ANOVA. [d]*p*-value: Welch's test.

tocopherols previously reported in corn grains.[8] Slightly higher concentrations for γ- and δ-tocotrienol were observed in corn grain samples from phosphate-fertilized plants compared to those samples without phosphate fertilization. α-Tocopherol was not detected. In a previously published report, the absence of α-tocopherol has been observed in leaves from *Arabidopsis thaliana* grown under severe phosphate deficiency.[47] The concentrations of γ- and δ-tocopherols and tocotrienols as well as the total tocochromanol concentrations were not significantly affected by phosphate fertilization in our experiment. A significant correlation between sowing time and phosphate fertilization was only observed for γ-tocopherol (p = 0.024) and total tocochromanols (p = 0.022). Besides concentration differences based on genetic variation, a significant ($p < 0.01$) interaction effect between genotype and the environment has been reported for γ-tocopherol contents in biofortified corn hybrids.[46]

■ CONCLUSIONS

In summary, differences in soil phosphate concentrations did not affect the concentrations of (poly)phenols, carotenoids, or tocochromanols in corn kernels. This may be attributed to the ability of the studied corn hybrid to adapt to low phosphate conditions. Plants have diverse phosphate recycling strategies to compensate for phosphate deficiency, e.g., by producing intracellular or extracellular acid phosphatase, increasing root hair density, or secreting organic acids into the rhizosphere.[48,49] This is supported by the observation that P fertilization in our experiment did not affect the yield or total P concentration of corn grains. Even in unfertilized controls, the total P concentrations of early- and late-sown corn grains were higher than the critical value (i.e., 2.8 mg P/g dry weight) used as an indicator for P deficiency in corn grains.[24] Based on these results, it appears that the grains in our study were sufficiently supplied with P in all treatments. With the exception of lutein, no significant correlations between grain P content and the concentration of (poly)phenols, carotenoids, and tocochromanols were found. In contrast, late sowing significantly increased the concentrations of *p*-coumaroyl-*N*′-feruloylputrescine, *N*,*N*′-diferuloylputrescine, lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and δ-tocotrienol, whereas early sowing increased insoluble *p*-coumaric acid contents. Future studies should investigate the impact of phosphate fertilization on corn grain (poly)phenols, carotenoids, and tocochromanols and processes in the rhizosphere from further corn genotypes, including contemporary hybrids and landraces, in order to reinforce the coherences described herein.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b07009.

(Table S1) Comparison of extraction yields of carotenoids from ground corn after different saponification times (0.5, 2, and 4 h) and at different temperatures (20, 38, and 70 °C); (Table S2) comparison of two different extraction solvents for the extraction of carotenoids from ground corn after saponification at 70 °C for 30 min with potassium hydroxide solution (50%, *w*/*v*); (Table S3) spike levels, recoveries, detection limits, quantitation limits, calibration linearity, and calibration range for soluble and insoluble (poly)-phenols and carotenoids in corn grains; and (Table S4) results of the tests for normality (Shapiro Wilk's test) and homoscedasticity (Levene's test) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; BHT, butylated hydroxytoluene; CAL-P, phosphate determined with the calcium acetate−lactate method; DAD, diode array detection; DW, dry weight; ESI-MS^n, electrospray ionization−multiple stage mass spectrometry; FLD, fluorescence detection; GDD, growing degree days; HPLC, high-performance liquid chromatography; HR-MS, high-resolution mass spectrometry; P, phosphorus; UV/vis, ultraviolet/visible light

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Supporting information

(Poly)phenols, Carotenoids, and Tocochromanols in Corn (*Zea mays* L.) Kernels as Affected by Phosphate Fertilization and Sowing Time

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Table S1. Comparison of extraction yields of carotenoids from ground corn after different saponification times (0.5, 2, and 4 h) and temperatures (20, 38, and 70 °C)

Saponification conditions	Concentration [µg/g]				
	Lutein	Zeaxanthin	β-Cryptoxanthin	α-Carotene	β-Carotene
20 °C, 2h	9.1 ± 0.6	5.3 ± 0.5	0.7 ± 0.2	0.1 ± 0	0.5 ± 0.2
20 °C, 4h	9.4 ± 0.4 [a]	6.4 ± 0.5 [a]	0.6 ± 0.1	0.1 ± 0	0.4 ± 0.1
40 °C, 2h	8.6 ± 0.5	6.2 ± 0.7	0.8 ± 0.2 [a]	0.1 ± 0	0.4 ± 0.1
70 °C, 0.5h	9.1 ± 0.4	6.3 ± 0.2	0.8 ± 0.0 [a]	0.2 ± 0 [a]	0.6 ± 0 [a]

Concentrations given as mean ± standard deviation (n = 3). [a] Maximum concentration within column.

Table S2. Comparison of two different extraction solvents for the extraction of carotenoids from ground corn after saponification at 70 °C for 30 min with potassium hydroxide solution (50%, *w*/*v*)

Extracting agent	Extraction steps [a]	Concentration [µg/g]				
		Lutein	Zeaxanthin	β-Cryptoxanthin	α-Carotene	β-Carotene
Hexane	1	6.8 ± 0.4	4.3 ± 0.3	0.7 ± 0.1	0.1 ± 0	0.5 ± 0.1
	2	9.1 ± 0.6	6.6 ± 0.6	1.0 ± 0.1	0.2 ± 0	1.0 ± 0.1
	3	9.8 ± 0.1	7.3 ± 0	1.1 ± 0.0	0.3 ± 0	1.3 ± 0.1
	4	10.8 ± 0.9	7.9 ± 0.6	1.2 ± 0.1	0.3 ± 0	1.5 ± 0.1
Hexane/ diethyl ether (50/50, *v*/*v*)	1	9.3 ± 0.5	7.0 ± 0.4	1.1 ± 0.8	0.3 ± 0	1.3 ± 0.1
	2	10.2 ± 0.7	7.6 ± 0.4	1.1 ± 0	0.3 ± 0	1.4 ± 0.1
	3	10.4 ± 0.1	7.8 ± 0.1	1.1 ± 0	0.3 ± 0	1.5 ± 0
	4	10.5 ± 0.9	7.8 ± 0.8	1.2 ± 0.1	0.4 ± 0	1.5 ± 0.1

[a] For each extraction step a solvent volume of 1 mL was used. Concentrations given as mean ± standard deviation (n = 3).

Table S3. Spike levels, recoveries, detection limits, quantitation limits, calibration linearity and calibration range for soluble and insoluble (poly)phenols and carotenoids in corn grains

Compound	Spike level [mg/L]		Recovery [%]		Detection limit [ng on column]	Quantitation limit [ng on column]	Correlation coefficient [-]	Regression line y = m · x + b	Calibration range [mg/L]
	High	Low	High	Low					
p-Coumaric acid (soluble)	12.0	0.6	99 ± 2	101 ± 7	0.37	1.12	0.9993	y = 108.17x + 48.42	0.1 – 100.0
Ferulic acid (soluble)	12.0	0.6	91 ± 2	97 ± 5	0.29	0.88	0.9999	y = 88.95 + 6.65	0.1 – 100.0
p-Coumaric acid (insoluble)	120.0	6.0	93 ± 4	88 ± 5	-	-	-	-	-
Ferulic acid (insoluble)	240.0	12.0	94 ± 6	91 ± 2	-	-	-	-	-
Lutein	4.0	0.2	90 ± 6	95 ± 7	0.05	0.16	0.9999	y = 1.234x + 0.006	0.08 – 6.00
Zeaxanthin	4.0	0.2	92 ± 3	95 ± 7	0.03	0.09	0.9998	y = 1.262x – 0.026	0.08 – 6.00
β-Cryptoxanthin	0.5	0.025	90 ± 1	97 ± 7	0.04	0.11	0.9995	y = 0.843x + 0.047	0.03 – 2.00
α-Carotene	0.5	0.025	92 ± 1	91 ± 5	0.05	0.13	0.9999	y = 0.961x – 0.015	0.03 – 2.00
β-Carotene	0.5	0.025	94 ± 1	93 ± 7	0.04	0.11	0.9995	y = 0.798x – 0.026	0.03 – 2.00

Recoveries represented as mean ± standard deviation ($n = 3$). Detection limits, quantitation limits, correlation coefficients and regression lines represented by means of three independent experiments. *b*: Y-intercept. *m:* Slope of the regression line. *x*: Peak area or ratio peak area / peak area internal standard; y = Concentration.

Table S4. Results of the tests for normality (Shapiro Wilk's test, $p < 0.05$) and homoscedasticity (Levene's test, $p < 0.01$)

Compound	*p*-values of Shapiro-Wilk's test				Levene's test	Suggested statistical test
	P0 (early)	P100 (early)	P0 (late)	P100 (late)	Whole data set	
N,N′-Di-*p*-coumaroylspermidine*	0.036	**0.652**	0.037	**0.308**	F(3,12) = 2,346; p = **0.12**	Kruskal-Wallis
p-Coumaric acid	**0.254**	**0.616**	0.015	**0.840**	F(3,12) = 2,502; p = **0.11**	One-way ANOVA
Ferulic acid	**0.934**	**0.063**	**0.083**	**0.182**	F(3,12) = 0,814; p = **0.51**	One-way ANOVA
N-*p*-Coumaroyl-*N′*-feruloylputrescine (2), *N,N′*-Diferuloylputrescine (2)**	**0.338**	**0.334**	0.038	**0.295**	F(3,12) = 4,061; ***p* = 0.03**	One-way ANOVA
Total soluble (poly)phenols	**0.743**	**0.051**	0.028	**0.341**	F(3,12) = 0,558; p = **0.65**	One-way ANOVA
8,8′-DiFA, aryltetralin form*	**0.314**	**0.488**	**0.371**	**0.540**	F(3,12) = 3,852; ***p* = 0.04**	One-way ANOVA
p-Coumaric acid	**0.851**	**0.696**	**0.068**	**0.626**	F(3,12) = 2,811; p = **0.09**	One-way ANOVA
Ferulic acid, 8,8′-DiFA*	**0.599**	**0.221**	**0.929**	**0.489**	F(3,12) = 1,269; p = **0.33**	One-way ANOVA

8,5′-DiFA*	**0.399**	**0.556**	**0.460**	0.031	F(3,12) = 2,089; p = **0.16**	One-way ANOVA
Triferulic acid (1)*	**0.240**	**0.072**	**0.480**	**0.349**	F(3,12) = 1,209; p = **0.35**	One-way ANOVA
5,5′-DiFA*	**0.487**	**0.217**	**0.742**	**0.495**	F(3,12) = 0,990; p = **0.43**	One-way ANOVA
8-*O*-4′-DiFA*	**0.101**	**0.142**	**0.203**	**0.690**	F(3,12) = 1,554; p = **0.25**	One-way ANOVA
8,5′-DiFA benzofuran form*	**0.321**	**0.219**	**0.774**	**0.483**	F(3,12) = 2,637; p = **0.10**	One-way ANOVA
Triferulic acid (2) *	**0.398**	**0.439**	**0.636**	**0.154**	F(3,12) = 4,680; ***p*** **= 0.02**	One-way ANOVA
Total insoluble (poly)phenols	**0.288**	**0.498**	**0.559**	**0.141**	F(3,12) = 2,539; p = **0.11**	One-way ANOVA
Lutein	0.004	**0.176**	**0.974**	**0.711**	F(3,44) = 1,360; p = **0.27**	One-way ANOVA
Zeaxanthin	**0.086**	**0.287**	**0.754**	**0.149**	F(3,44) = 9,426; $p \leq 0,01$	Welch's test
β-Cryptoxanthin	**0.130**	**0.052**	**0.177**	**0.232**	F(3,44) = 1,313; p = **0.28**	One-way ANOVA
α-Carotene	**0.066**	**0.106**	0.028	**0.093**	F(3,44) = 2,147; p = **0.11**	One-way ANOVA
β-Carotene	**0.648**	**0.482**	**0.168**	**0.159**	F(3,44) = 0,213; p = **0.89**	One-way ANOVA
δ-Tocotrienol	≤ 0.001	**0.703**	0.005	**0.061**	F(3,44) = 4,778; p = **0.01**	Kruskal-Wallis
γ-Tocotrienol	0.016	0.003	**0.215**	**0.371**	F(3,44) = 0,968; p = **0.42**	Kruskal-Wallis
δ-Tocopherol	0.007	0.007	0.002	0.001	F(3,44) = 0,958; p = **0.42**	Kruskal-Wallis
γ-Tocopherol	**0.600**	**0.453**	**0.052**	0.005	F(3,44) = 1,784; p = **0.16**	One-way ANOVA
Total carotenoids	0.027	**0.093**	**0.613**	**0.148**	F(3,44) = 1,397; p = **0,25**	One-way ANOVA
Total tocochromanols	**0.303**	**0.164**	**0.991**	0.004	F(3,44) = 1,215; p = **0,32**	One-way ANOVA

Chapter 3

Location and variety but not phosphate starter fertilization influence the profiles of fatty acids, carotenoids, and tocochromanols in kernels of modern corn (*Zea mays* L.) hybrids cultivated in Germany

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Location and Variety but Not Phosphate Starter Fertilization Influence the Profiles of Fatty Acids, Carotenoids, and Tocochromanols in Kernels of Modern Corn (*Zea mays* L.) Hybrids Cultivated in Germany

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ABSTRACT: Phosphate is a limiting plant nutrient and essential for corn growth and development. Thus, the impact of phosphate fertilization, location, and the variety of modern corn (*Zea mays* L.) hybrids on the profiles of fatty acids, carotenoids, and tocochromanols (vitamin E) was assessed in corn grains. Eight different corn hybrids were grown with (52.9 kg of phosphorus per ha) or without starter fertilizer at three experimental sites in Germany. Location ($p < 0.05$) and genetics ($p < 0.001$) but not phosphate fertilization significantly altered the concentrations of individual saturated and unsaturated fatty acids, carotenoids, and tocochromanols. Significant ($p < 0.05$) interaction effects on the concentrations were mainly observed between the variety and the location. In conclusion, the choice of the corn variety had a more significant impact on the biosynthesis of fatty acids, carotenoids, and tocochromanols than the location or phosphate application on phosphate-sufficient soils.

KEYWORDS: *corn grain, phosphate availability, fatty acid, vitamin E, carotenoid*

INTRODUCTION

Phosphorus (P) is one of the most limiting macronutrients for plants.[1] Within plant cells, P is involved in the synthesis of nucleic acids, ATP production, and redox reactions.[2] To enhance phosphate availability in soils and to stabilize growing performance in crop production, phosphate fertilizers are applied in agriculture.[3] However, finite global phosphate resources and eutrophication of freshwater demand optimized and sustainable fertilization strategies.[4−6] Facing these global challenges, the European Commission has targeted the reduction in fertilizer use within the European Union by at least 20% until 2030.[7] At the same time, the production of safe and nutritious foods must be secured, but effects of a reduced phosphate availability on the biosynthesis of secondary plant metabolites, fatty acids, and vitamins in corn have rarely been investigated. Especially, polyunsaturated fatty acids (e.g., linoleic acid), vitamin A, in the form of provitamin A carotenoids, and α-tocopherol (vitamin E) in corn grains are important lipophilic nutrients for humans,[8] and their accumulation in the grains may rely on the availability of phosphate.

In the biosynthetic pathway of tocochromanols in corn, phosphate is required for the formation of the intermediates phytyl diphosphate and geranylgeranyl diphosphate.[9] The latter is also an intermediate in the carotenoid synthesis. Further dimerization of geranylgeranyl diphosphate, desaturation, cyclization, and isomerization yield carotenes (α- and β-carotene) and, after hydroxylation, xanthophylls (lutein, zeaxanthin, β-cryptoxanthin).[10] In fatty acid synthesis in plastids of corn embryos, phosphate is involved as adenosine triphosphate and the reducing equivalent nicotinamide adenine dinucleotide phosphate.[11]

Changing climatic conditions pose another challenge that requires adaption by plants and may influence their profiles of fatty acids, carotenoids, and tocochromanols even within the temperate region. Extreme weather events, such as drought, limit growth and cause oxidative damage in plant cells.[12] In addition, reduced amounts of total provitamin A carotenoids were found in corn grains from plants grown under drought in comparison to a control group.[13] On the contrary, a low growth temperature (10 °C) did not reduce carotenoid concentrations of sweet corn seedlings and even increased vitamin E concentrations.[14] It should also be noted that the mobilization and uptake of phosphates from the rhizosphere are dependent on environmental factors and soil properties, such as precipitation and soil pH.[15]

Thus, we hypothesized that phosphate availability and location mediate alterations in the profiles and concentrations of fatty acids, carotenoids, and tocochromanols (vitamin E) in different corn hybrid varieties. Furthermore, interaction effects between phosphate fertilization, the location, and the variety on the concentrations of these lipophilic compounds were analyzed

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in-depth. To the best of our knowledge, this is the first study considering three-way interaction effects between phosphate fertilization, location, and variety on fatty acids, carotenoids, and vitamin E in corn using a panel of eight varieties and three locations.

MATERIALS AND METHODS

Chemicals. L(+)-Ascorbic acid (purity ≥ 99%), butylated hydroxytoluene (BHT), 1,4-dioxane, ethanol, *n*-hexane, potassium hydroxide solution (50%, w/v), glacial acetic acid, sodium chloride (purity ≥ 99%), and sulfuric acid (purity ≥ 95%) were purchased from Carl Roth (Karlsruhe, Germany). (all-*E*)-*β*-*Apo*-8′-carotenal (purity ≥ 96%), diethyl ether (≥99%, inhibitor-free), ethyl myristate, analytical standard mixture containing 10 saturated or unsaturated fatty acid methyl esters (FAME; C_{14} to C_{22}), methoxyamine hydrochloride (both purity ≥ 98%), *α*-carotene, *α*-, *β*-, *γ*-, *δ*-tocopherol and -tocotrienol standards (purity ≥ 95.5%) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Acetonitrile and methanol were obtained from J.T. Baker (Gliwice, Poland). Lutein, zeaxanthin, *β*-cryptoxanthin, and *β*-carotene (purity at least 99%) were ordered from Extrasynthèse (Genay, France). Purified deionized water was prepared by a Milli-Q (Millipore, Billerica, MA) water treatment system. All solvents and reagents were at least of high-performance liquid chromatography (HPLC) grade.

Plant Material and Field Trials. Replicated field experiments with eight yellow corn (*Zea mays* L.) hybrids were carried out at three different locations in a randomized complete block design in 2019. Corn hybrids with early to medium−early maturity (K220 to K250) were selected from the official list of varieties by the Federal Plant Variety Office in Germany.[16] The chosen hybrids comprised "Amaveritas" (Agromais, Everswinkel, Germany; maturity number K240), "ES Metronom" (Euralis Saaten, Norderstedt, Germany; K240), "Figaro" (K250), "Ricardinio" (both KWS SAAT, Einbeck, Germany; K220), "Hulk" (agaSAAT, Neukirchen-Vluyn, Germany; ∼K250), "LG 30.258" (Limagrain, Edemissen, Germany; K240), "P8329" (Corteva Agriscience, Munich, Germany; K240), and "SY Talisman" (Syngenta, Bad Salzuflen, Germany; K230). The experimental sites were at Heidfeldhof nearby Stuttgart-Hohenheim (48°43′05.7″N, 9°11′20.8″E; altitude, 409 m; annual mean temperature in 2019, 10.6 °C; annual precipitation, 856.5 mm; soil texture, silty loam), Eckartsweier (48°32′24.7″N, 7°51′15.1″E; altitude, 136 m; annual mean temperature in 2019, 11.7 °C; annual precipitation, 782.8 mm; soil texture, clayey loam), and Dettingen (48°35′32.3″N, 10°08′23.4″E; altitude, 563 m; annual mean temperature in 2019, 9.1 °C; annual precipitation, 661.4 mm; soil texture, clayey loam). Additional details on soil characteristics are given in Table S1.

The field trials were sown in an alpha lattice design and grown under standard agronomical practices. Two different treatments of starter fertilizer (0 or 52.9 kg P/ha) were applied. Each hybrid-treatment combination was replicated twice. One plot consisted out of two rows and amounted to 7.5 m^2. Corn grains were harvested at full maturity using a combine harvester.

Sample Preparation. The grains were dried at 40 °C to obtain a final moisture content below 14% and storability of the grains. The dried samples were transported to the laboratory in Hohenheim and stored at 4 °C until grinding. Whole corn grains from each plot were pre-ground (Rotor GT 800, Rotor, Uetendorf, Switzerland) and milled to a particle size of $d_p \leq 500$ *μ*m (Fritsch Pulverisette 14, Fritsch, Idar-Oberstein, Germany). The resulting corn flour was packed into resealable containers, head-space flushed with nitrogen, and stored at −80 °C in the dark until analysis.

Analyses of Fatty Acids, Carotenoids, and Tocochromanols. *Fatty Acid Distribution by Gas Chromatography Flame Ionization Detector (GC-FID) and Gas Chromatograph-Electron Impact-Mass Spectrometer (GC-EI-MS).* Fatty acids were transesterified and extracted as fatty acid methyl esters (FAME) following a modified protocol.[17,18] An aliquot of 12 mg of ground corn sample was mixed in a centrifuge tube with 3 mL of 1% sulfuric methanol. The suspension was heated in a covered water bath (80 °C, 4 h) and was sonicated after 1 h for 10 min. The sample was cooled on ice. Subsequently, 1 mL of ultrapure water and 1 mL of a saturated sodium chloride solution were admixed. FAME were extracted with 8 mL of *n*-hexane. A subsample was transferred together with ethyl myristate (0.2 mg/mL) as internal standard into an amber glass vial for gas chromatography (GC) analysis.

FAME were analyzed by a 5890 Series II gas chromatograph (Hewlett-Packard/Agilent, Waldbronn, Germany) equipped with a flame ionization detector (FID) and a 7673 injection system operating in splitless mode. Separation was achieved with an RTX 2330 column (60 m × 0.25 mm id, 0.1 *μ*m film thickness; Restek, Bellefonte, PA) coated with 90% biscyanopropyl and 10% phenylcyanopropyl polysiloxane. The injection volume was 1 *μ*L and the injector temperature was held at 250 °C. The carrier gas was nitrogen (99.999% purity) at a flow rate of 1.3 mL/min. The following temperature program was used: Isothermal at 60 °C (1 min), 60−150 °C (6 °C/min), 150−190 °C (3 °C/min), 190−250 °C (6 °C/min) and isothermal at 250 °C (5 min). A representative chromatogram of FAME in corn grain samples after transesterification is shown in Figure S1. Peaks were assigned to individual FAME by comparing the retention times with those of authentic reference standards. Quantitation of FAME was performed with external calibration curves of FAME. Composition of FAME was expressed as a percentage of total FAME in the grains.

The FAME profile of each variety was confirmed by a 5890 Series II Plus gas chromatograph coupled to a 5972 Series mass selective detector (MS; Hewlett-Packard/Agilent). Electron ionization (EI) was used as an ionization source. The above-described injection settings, column, and temperature program were applied. The transfer line temperature was 270 °C. Helium (99.999% purity) at a flow rate of 1 mL/min was used as a carrier gas. The scan range was set to m/z 50−550. Peaks were assigned to individual FAME based on retention times and mass spectra in comparison to reference standards. Identities of saturated FAME were confirmed by means of low abundant molecular ion $[M]^+$ and the characteristic fragment ion m/z 74 formed by McLafferty rearrangement. Oleic and linoleic acid methyl ester were identified by the loss of the McLafferty ion resulting in the fragment $[M - 74]^{+}$[19] and by the abundance ratio of fragment ions m/z 74, 79, 81, and 87 (for monounsaturated FAME: 74 > 87 > 81 > 79, and for diunsaturated FAME: 81 > 79).

Extraction and HPLC-UV/Vis Analyses of Carotenoids. Carotenoid extraction and HPLC analysis were performed according to a previously validated method.[20] Briefly, 100 mg of ground corn was saponified (30 min, 70 °C) in an ethanolic potassium hydroxide solution containing BHT as antioxidant and *β*-*apo*-8′-carotenal-methyloxime as internal standard. The solution was subsequently cooled on ice to reduce thermal degradation during the subsequent extraction and diluted with sodium chloride solution (15%, w/v). Glacial acetic acid was added. Carotenoids were extracted twice with a binary mixture of *n*-hexane/diethyl ether (50/50, v/v). The solution was vortexed, centrifuged (3 min, 140*g*, 4 °C) and the organic layer collected in a centrifuge tube. The combined organic layers were evaporated to dryness in a centrifugal concentrator (10 mbar, 20 °C). The extract was dissolved in 300 *μ*L of mobile phase and injected into the Shimadzu (Kyoto, Japan) Prominence HPLC-UV/Vis (450 nm) instrument. Figure S2 shows a representative chromatogram of carotenoids in ground corn grain extracts. Quantitation was examined by calibration curves of individual xanthophylls (lutein, zeaxanthin, *β*-cryptoxanthin) and carotenes (*α*- and *β*-carotene) within the quantitation range. Total provitamin A carotenoids were determined by the sum of *β*-cryptoxanthin and carotene concentrations. Total carotenoids comprised xanthophylls and carotenes.

Extraction and HPLC-FLD Analyses of Tocochromanols (Vitamin E). Tocopherols and tocotrienols were extracted and analyzed according to previously published methods.[20,21] In brief, 50 mg of ground corn was admixed with ultrapure water, potassium hydroxide solution (50%, w/v), and ethanol containing ascorbic acid (1%, w/v). The suspension was saponified in a shaking water bath (30 min, 70 °C) and cooled on ice. BHT (1 mg/mL), glacial acetic acid, and ultrapure water were added following exhaustive extraction with *n*-hexane. The combined organic phases were evaporated in a vacuum concentrator

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Table 1. *P*-values (Significant *P*-Values Highlighted in Bold) of Phosphate Fertilization, Location, Variety, and Their Interaction Effects on the Fatty Acid Composition in Corn Grains Using a Mixed Model[a]

	P-value[a]							
fatty acid	phosphate fertilization[b]	variety[c]	location[c]	phosphate fertilization × variety[c]	phosphate fertilization × location[c]	variety × location[c]	phosphate fertilization × variety × location[c]	repetition (plot)[c]
palmitic acid (16:0)	0.75	**<0.001**	1.00	1.00	1.00	**<0.001**	1.00	1.00
stearic acid (18:0)	0.79	**<0.001**	**0.03**	1.00	1.00	**0.04**	1.00	0.58
arachidic acid (20:0)	0.07	**<0.001**	0.56	1.00	1.00	**0.02**	0.64	0.57
oleic acid (18:1*n*−9)	0.69	**<0.001**	**0.02**	1.00	0.30	**<0.01**	0.64	1.00
linoleic acid (18:2*n*−6)	0.87	**<0.001**	**0.02**	1.00	0.40	**0.03**	0.22	1.00
α-linolenic acid (18:3*n*−3)	0.69	**<0.001**	**0.01**	1.00	1.00	**<0.001**	1.00	1.00
total saturated fatty acids	0.39	**<0.001**	0.82	0.74	1.00	**<0.001**	1.00	1.00
total unsaturated fatty acids	0.39	**<0.001**	0.82	0.74	1.00	**<0.001**	1.00	1.00

[a]Statistical significance stated at $p < 0.05$. [b]Tested as fixed effect, Wald-*F*-test. [c]Tested as random effect, loglikelihood ratio test.

Table 2. Fatty Acid Composition of Grains from Corn Hybrids Grown at the Three Locations Hohenheim, Eckartsweier, and Dettingen in Germany

	fatty acid composition (%)[a,d]		
fatty acid	Hohenheim	Eckartsweier	Dettingen
palmitic acid (16:0)[b]	12.8a (12.2 − 14.2)	12.6a (12.2 − 13.7)	13.0a (12.4 − 13.6)
stearic acid (18:0)[c]	1.8 ± 0.2b	2.0 ± 0.2a	1.7 ± 0.2b
arachidic acid (20:0)[b]	0.9a (0.8 − 1.0)	1.0a (0.9 − 1.1)	0.9a (0.8 − 1.1)
oleic acid (18:1*n*−9)[b]	25.4b (25.0 − 26.5)	28.5a (26.9 − 29.4)	24.3c (23.1 − 25.1)
linoleic acid (18:2*n*−6)[c]	57.9 ± 2.7a	55.5 ± 2.5b	59.4 ± 2.4a
α-linolenic acid (18:3*n*−3)[b]	0.6ab (0.6 − 0.7)	0.6b (0.6 − 0.7)	0.7a (0.6 − 0.7)
total saturated fatty acids[b]	15.7a (14.9 − 16.8)	15.6a (15.2 − 16.8)	15.7a (15.2 − 16.3)
total unsaturated fatty acids[b]	84.3a (83.2 − 85.1)	84.4a (83.2 − 84.8)	84.3a (83.7 − 84.8)

[a]Composition in the grains of eight corn varieties grown each in four plots was represented as mean ± standard deviation or median (interquartile range). Concentrations displaying different letters (a, b, c) show significant differences between the locations at $p < 0.05$. [b]Analyzed by pairwise Kruskal−Wallis test. [c]Analyzed by one-way ANOVA with Bonferroni post hoc test. [d]Expressed as a percentage of fatty acid methyl esters.

(10 mbar, 20 °C). Extracts were dissolved in 300 μL of ethanol and analyzed by HPLC-FLD (Shimadzu, Kyoto, Japan). A representative chromatogram of tocochromanols in ground corn grain extracts is shown in Figure S3. The samples were quantitated using linear calibration curves of α-, β-, γ-, δ-tocopherols and -tocotrienols. Total tocochromanols were calculated by the sum of the individual tocopherol and tocotrienol concentrations.

Method Validation. Precision of the extraction methods was verified at 100% of the test concentration of corn variety P8329 with six determinations within 1 day (intraday repeatability) and six determinations on 3 days over a 3-week period (interday repeatability) in accordance with the guidelines of the International Council for Harmonization (ICH).[22] Repeatabilities were summarized in Table S2.

Statistical Analyses. Data analyses of the traits (phosphate fertilization, location, variety) and their corresponding interaction effects were performed using the following statistical model

$$y_{ijkl} = \mu + t_i + v_j + l_k + (tv)_{ij} + (tl)_{ik} + (vl)_{jk} + (tvl)_{ijk} + r_{ikn} + \varepsilon_{ijkn} \quad (1)$$

where each trait value of the *i*th phosphate starter fertilizer application, the *j*th variety in the *k*th location and the *n*th replicate within the *k*th location and *i*th phosphate fertilizer application was represented by y_{ijkl}. μ denotes the overall mean and t_i denotes the fixed effect of the *i*th fertilizer application; the effect of the *j*th variety was characterized by v_j, and the effect of the *k*th location was defined by l_k; all two-way factor interactions were represented as $(tv)_{ij}$, $(tl)_{ik}$, and $(vl)_{jk}$; $(tvl)_{ijk}$ describes the corresponding three-way interaction. The replicate effect of each fertilization treatment within each location was defined by r_{ikn}. The residual term was characterized by ε_{ijkn}. The statistical software R (R Foundation for Statistical Computing, 2020, available at www.r-project.org) and the software ASRemL[23] were used for the calculation of the mixed model.

Differences in the profiles of fatty acids, carotenoids, and tocochromanols between the locations or between the varieties were assessed as follows: Shapiro Wilk's test and Levene's test (both $p < 0.05$) were conducted for each dataset to check for normality and homogeneity of variances. Composition and concentration were represented as mean ± standard deviation or median with 25 and 75% percentile. Significant differences between the individual groups were assessed by one-way analysis of variance (ANOVA) with Bonferroni post hoc test (parametric test) or pairwise Kruskal−Wallis test with Bonferroni correction (nonparametric test) using SPSS version 25 (IBM, Armonk, NY). Statistical significance was stated at a $p < 0.05$.

RESULTS AND DISCUSSION

Fatty Acid Profiles. *Effects of Phosphate Fertilization.* The fatty acids in the corn grains, determined as FAME by GC-EI-MS, comprised saturated (palmitic, stearic, arachidic), mono-

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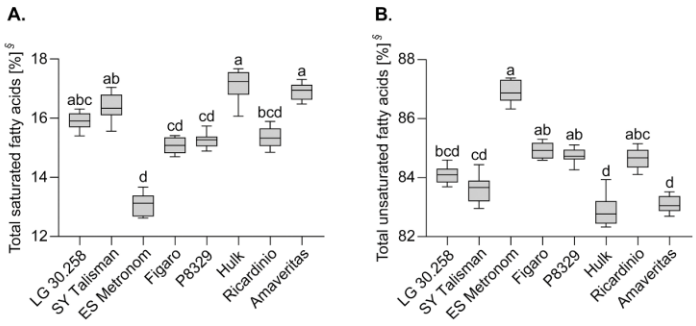

Figure 1. Boxplots of total saturated (A) and total unsaturated fatty acids (B) in the grains of eight corn varieties. Medians were calculated based on the concentrations in the grains of each variety grown at the three sites in four plots. Different letters represent significant ($p < 0.05$) differences between medians. §Fatty acids expressed as a percentage of total saturated or total unsaturated fatty acid methyl esters. The reader should be aware of the different scales of the ordinates.

unsaturated (oleic), and polyunsaturated (linoleic, α-linolenic) fatty acids. In the grains of all samples, linoleic acid contributed more than half of the total fatty acids, whereas arachidic and α-linolenic acid were present in minor proportions (< 3%). The identified saturated and unsaturated fatty acids were consistent with literature data.[24−26] In terms of phosphate fertilization, the fatty acid profile in the grains from corn plants grown with or without phosphate application remained almost constant (Table S3) and was not significantly affected (Table 1). On the contrary, an increase in the share of unsaturated fatty acids accompanied by a decrease in saturated fatty acids in the grains has been reported after fertilizing corn plants with a combination of phosphorus, potassium, and nitrogen fertilizer.[27] In this study, potassium appeared to have a larger impact on oil quality than nitrogen or phosphorus. Although the uptake of phosphate from the rhizosphere is dependent on factors such as soil pH or genotype-specific root morphology,[15,28] interaction effects between phosphate fertilization and the location or between phosphate fertilization and the variety on the fatty acid profile in the corn grains were also not significant. Notably, some plants are able to compensate changes in phosphate availability to a certain extent even within a plant cell. For instance, in soybean leaves, phosphorus stored in vacuoles is preferentially remobilized into the cytosol to maintain metabolism at the R2, R4, and R6 stages under reduced phosphate availability.[29] Apparently, fatty acid composition in the grains of the eight modern corn hybrids did not depend on the application of phosphate fertilizer on well-supplied soils.

Locational Influences. The location (place of cultivation comprising multifactorial climatic conditions and soil properties) significantly affected the fatty acid profile of the corn grains (Table 2). In particular, the proportion of stearic acid (18:0), oleic acid (18:1n−9), linoleic acid (18:2n−6), and α-linolenic acid (18:3n−3) in the grains differed among the locations. The highest proportions in stearic acid (2.0%) and oleic acid (28.5%) were found in grains from plants grown at Eckartsweier. Significantly lower proportions in oleic acid were observed in the grains from Hohenheim (25.4%) and Dettingen (24.3%). Accompanied by a decrease in oleic acid, significantly higher proportions in linoleic acid were observed in grains from Dettingen (59.4%) and Hohenheim (57.9%) compared to Eckartsweier (55.5%). Maximum proportions of α-linolenic acid were present in the grains from Dettingen with 0.7%. In plants, fatty acids are involved in abiotic stress responses, for instance, to maintain membrane fluidity during fluctuating temperatures.[30] It is assumed that high growing temperatures, especially at night, are responsible for a decrease in linoleic acid and an accumulation of oleic acid due to the temperature-dependent activity of the desaturase.[31] In corn, an upregulation of the genes encoding for fatty acid desaturases (e.g., *ZmFAD2.1* and *ZmSLD1*-3) were observed under cold stress, whereas these genes were downregulated under high temperatures.[32]

In our experiment, the annual mean temperatures were indeed different among the experimental sites, with the lowest mean temperature observed in Dettingen (9.1 °C), followed by Hohenheim (10.6 °C) and Eckartsweier (11.7 °C). These findings imply that the temperature may have influenced the biosynthetic pathway, in particular the expression profile or activity of desaturases, resulting in the significantly higher proportion of linoleic acid and α-linolenic acid and lower proportion of oleic acid in the grains from Dettingen in comparison to the grains from Eckartsweier. However, a combination of many different environmental factors, including temporary stressors, may impact the fatty acid composition and impede a clearer explanation.[30] Effects of the temperature on the desaturation of fatty acids were also observed for certain fish species showing a higher proportion of unsaturated fatty acids for fish living in Arctic waters (9 °C) than in tropical surface water (23−25 °C).[33]

Although the described changes in the proportion of mono- and polyunsaturated fatty acids in the grains may be too small to have an impact on human health after ingestion, the fatty acid composition could affect the storage stability of the grains since the degree of unsaturation of fatty acids influences the formation of primary and secondary oxidation products.[34] A lower oxidative stability was also observed for corn oil with 24% of oleic acid and 63% of linoleic acid revealing significantly higher peroxide values than a high-oleic corn oil containing about 65% of oleic acid and 23% linoleic acid under accelerated storage conditions at 60 °C for 8 days.[35]

Compositional Differences between Corn Varieties. The factor variety most strongly altered the fatty acid composition in comparison to phosphate fertilization or the location ($p < 0.001$; Table 1). The median proportion of total saturated fatty acids in the grains ranged from 13.1% for the corn variety ES Metronom to 17.2% for Hulk (Figure 1). The proportion of total saturated fatty acids of the corn samples presented in this study were similar to those reported for rye (16.4%) and peas (14.7%), but above the proportion reported for mustard (4.9%).[36] The varieties Hulk, Amaveritas (16.9%), and SY Talisman (16.3%)

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had significantly higher proportions of total saturated fatty acids than ES Metronom. The reversed order was observed for the proportion of unsaturated fatty acids with a maximum proportion of 86.9% for ES Metronom and a minimum proportion of 82.8% for Hulk. In general, all grains of the corn varieties examined had a high proportion (> 82%) of unsaturated fatty acids. For corn oil production, the fatty acid composition and the position of the fatty acid in the triacylglycerols are the main determinants of their physical properties, such as the melting point. Although hydrogenation or interesterification of fatty acids is widely accepted in the food industry to control the melting behavior of oils and fats,[37] information about the fatty acid composition of the different varieties may be useful for food producers to reduce processing steps by specifically selecting the corn variety.

Carotenoid Profiles. *Effects of Phosphate Fertilization.* The xanthophyll (lutein, zeaxanthin, β-cryptoxanthin) and carotene (α- and β-carotene) profiles were similar in all corn samples studied. Phosphate fertilization did not significantly affect the concentration of these carotenoids (Table 3), which is in line with a previous work under low plant-available phosphate conditions.[20] Synergistic effects between phosphate fertilization and the location or between phosphate fertilization and the variety on carotenoid concentrations were not significant. At present, these results suggest that a reduction of phosphate fertilization is possible for modern corn hybrids without altering the carotenoid concentrations in the grains. In another trial, nitrogen fertilization in combination with tillage significantly increased the concentrations of total carotenoids (sum of lutein, zeaxanthin, and β-carotene), reaching concentrations of up to 36.9 μg/g.[38] In a field study conducted with soybeans, a marginal increase of 4% in the lutein content was demonstrated after fertilizing the plants with 50 kg of phosphorus per hectare.[39] Comparing these studies with our results, nitrogen fertilizer and tillage appear to have a greater influence on carotenoid concentrations than phosphate starter fertilization. Nevertheless, significant effects of phosphate fertilization in combination with locational factors and variety-dependent effects on β-cryptoxanthin and total provitamin A carotenoids were identified, but will require further investigations.

Locational Influences. The concentrations of individual provitamin A carotenoids were affected by the location (Tables 3 and 4). Higher concentrations of total provitamin A carotenoids were found in corn grains from Eckartsweier (5.2 μg/g dry weight, DW) compared to grains from Hohenheim (4.5 μg/g DW) and Dettingen (4.3 μg/g DW). The changes in total provitamin A carotenoids were mainly attributed to the concentration of β-cryptoxanthin, with the highest median concentrations of 2.3 μg/g DW in the grains from Eckartsweier and lowest median concentrations in the grains from Dettingen with 1.7 μg/g DW. As observed for proportions in stearic acid and oleic acid, a positive trend in the concentrations of total provitamin A carotenoids toward higher mean temperatures at the sites was observed, suggesting a dependency on the temperature. Significant variation in the carotenoid contents, assumingly due to changing air temperatures or rainfall, has been reported in a previous study.[40] Besides temperature, light intensity is another factor that can promote the accumulation of plant pigments, as observed in chloroplasts at temperatures between 10 and 14 °C.[41] Interestingly, median concentrations of total carotenoids remained almost constant, ranging from 34.6 μg/g DW in the grains from corn plants grown in Dettingen to 36.7 μg/g DW in the grains from Hohenheim. Significant

Table 3. *P*-Values (Significant *P*-Values Highlighted in Bold) of Phosphate Fertilization, Location, Variety, and Their Interaction Effects on Carotenoid Concentrations in Corn Grains Using a Mixed Model

	P-value[a]							
carotenoid	phosphate fertilization[b]	variety[c]	location[c]	phosphate fertilization × variety[c]	phosphate fertilization × location[c]	variety × location[c]	phosphate fertilization × variety × location[c]	repetition (plot)[c]
lutein	0.68	**<0.001**	0.46	1.00	0.19	**<0.001**	1.00	1.00
zeaxanthin	0.06	**<0.001**	0.66	1.00	1.00	**<0.001**	0.75	1.00
β-cryptoxanthin	0.40	**<0.001**	**0.02**	1.00	0.86	**<0.001**	**0.01**	1.00
α-carotene	0.46	**<0.001**	0.05	1.00	1.00	**<0.001**	1.00	1.00
β-carotene	0.90	**<0.001**	**0.02**	0.43	1.00	**<0.001**	0.10	0.99
total provitamin A carotenoids	0.61	**<0.001**	**0.01**	1.00	1.00	**<0.001**	**0.03**	0.89
total carotenoids	0.22	**<0.001**	0.88	1.00	0.68	**<0.001**	0.89	1.00

[a]Statistical significance stated at $p < 0.05$. [b]Tested as fixed effect, Wald-*F*-test. [c]Tested as random effect, loglikelihood ratio test.

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Table 4. Carotenoid Concentrations of Grains from Corn Plants Grown at the Three Locations Hohenheim, Eckartsweier, and Dettingen in Germany

	concentration (μg/g DW)[a]		
carotenoid	Hohenheim	Eckartsweier	Dettingen
lutein[b]	20.1a (18.9–26.4)	19.6a (16.5–24.0)	19.8a (16.3–25.2)
zeaxanthin[b]	11.8a (10.3–14.8)	11.5a (10.2–13.7)	12.9a (10.4–14.4)
β-cryptoxanthin[b]	1.8ab (1.3–2.3)	2.3a (1.8–2.5)	1.7b (1.3–1.9)
α-carotene[b]	0.7ab (0.4–0.9)	0.8a (0.6–1.1)	0.7b (0.4–0.7)
β-carotene[b]	2.0a (1.6–2.8)	2.1a (1.9–3.5)	2.0a (1.7–2.5)
total provitamin A carotenoids[b]	4.5b (3.9–5.5)	5.2a (4.8–6.6)	4.3b (3.8–4.8)
total carotenoids[b]	36.7a (32.8–42.2)	36.3a (33.2–41.4)	34.6a (33.4–41.4)

[a]DW, dry weight. Concentration in the grains of eight corn varieties grown each in four plots was represented as median (interquartile range). Concentrations displaying different letters (a, b) show significant differences between the locations at $p < 0.05$. [b]Analyzed by pairwise Kruskal–Wallis test.

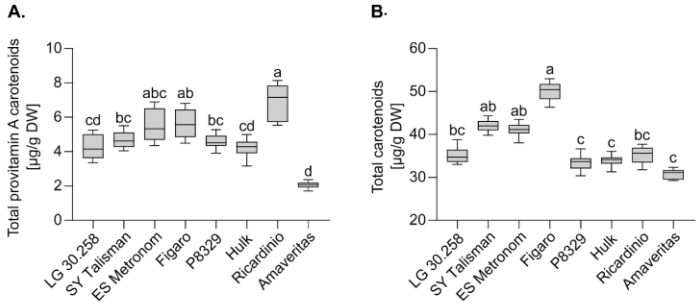

Figure 2. Boxplots of total provitamin A carotenoids (A) and total carotenoids (B) in the grains of eight corn varieties. Medians were calculated based on the concentrations in the grains of each variety grown at the three sites in four plots. Different letters represent significant ($p < 0.05$) differences between medians.

interaction effects between the location and the variety were observed for all carotenoids present in the corn samples. This observation supports the hypothesis that variations in the metabolome of corn grains are dependent on the genotype and even on slight changes in the environmental conditions on the field.[42]

Concentration Differences between Corn Varieties. Carotenoid concentrations in the grains varied significantly between the studied varieties, ranging from 2.1 to 7.2 μg/g DW for total provitamin A carotenoids and 31.1–50.4 μg/g DW for total carotenoids (Figure 2). The highest concentration in provitamin A carotenoids were found in the grains of Ricardinio (7.2 μg/g DW). In general, high concentrations in provitamin A carotenoids in corn are desired and biofortification strategies to eradicate vitamin A deficiency, especially in Sub-Saharan countries, aimed at 15 μg/g of retinol equivalents in the HarvestPlus breeding program.[43]

Regarding total carotenoids, significantly higher concentrations were found in grains from Figaro (50.4 μg/g DW), SY Talisman (42.0 μg/g DW), and ES Metronom (41.1 μg/g DW) in comparison to the concentrations in Hulk (34.1 μg/g DW), P8329 (33.7 μg/g DW), and Amaveritas (31.1 μg/g DW). Interestingly, high concentrations in total carotenoids were not always accompanied by high concentrations in total provitamin A carotenoids as observed in the grains from SY Talisman with high concentrations in total carotenoids (42.0 μg/g DW), but moderate concentrations in provitamin A carotenoids (4.6 μg/g DW). In general, the concentrations of total carotenoids in the grains of the hybrids were within the expected range for corn of 1.1–61.1 mg/kg DW described by Berardo et al.,[44] but above the range of 0.2–33.1 μg/g DW reported by Kurilich and Juvik.[45] Total carotenoid concentrations in the corn grains were higher than in einkorn grains (5.3–13.6 μg/g DW),[46] but lower than total carotenoids found in vacuum dried broccoli (459.0 μg/g DW) or carrots (989.0 μg/g DW).[47] The concentrations of provitamin A carotenoids in the corn grain samples given as retinol activity equivalents ranged from 11.4 μg/100 g in Amaveritas to 45.6 μg/100 g in Ricardinio. Thus, 5.4% (men) to 6.5% (women) of the daily allowance in vitamin A would be covered by the consumption of 100 g of grains from Ricardinio, when considering the recommendations for vitamin A of the German Nutrition Society.[48] Notably, postharvest processing and storage conditions also have an impact on the concentrations of carotenoids in corn grains. In addition, the degradation kinetics of carotenoids in the corn grains are supposed to be dependent on the genotype,[49] making a detailed comparison with literature data even more complex.

Tocochromanol Profiles. *Effects of Phosphate Fertilization.* For the total tocochromanol concentrations in the grains of the corn hybrids, neither phosphate fertilization nor interaction effects between phosphate fertilization and the location or the variety were significant (Table 5). This was also observed for the individual tocopherols and tocotrienols with the exception of β-tocopherol, which was significantly affected by the variety in combination with phosphate fertilization. As observed for carotenoids, the accumulation of tocochromanols in the corn grains appeared to be unaffected by phosphate fertilization even under conditions of low plant-available phosphate.[20] Furthermore, the largest increase in tocochromanols in the grains occurred in the later stages of grain filling, shortly before the

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Table 5. *P*-Values (Significant *P*-Values Highlighted in Bold) of Phosphate Fertilization, Location, Variety, and Their Interaction Effects on Tocochromanols in Corn Grains Using a Mixed Model

	P-value[a]							
tocochromanol	phosphate fertilization[b]	variety[c]	location[c]	phosphate fertilization × variety[c]	phosphate fertilization × location[c]	variety × location[c]	phosphate fertilization × variety × location[c]	repetition (plot)[c]
α-tocopherol	0.16	**<0.001**	1.00	1.00	1.00	**0.01**	1.00	1.00
β-tocopherol	0.52	**<0.001**	0.10	**<0.01**	0.84	**<0.01**	1.00	1.00
γ-tocopherol	0.34	**<0.001**	**0.04**	1.00	1.00	0.05	1.00	1.00
δ-tocopherol	0.76	**<0.001**	**0.01**	1.00	1.00	**<0.001**	1.00	1.00
α-tocotrienol	0.25	**<0.001**	0.24	1.00	1.00	**0.04**	1.00	1.00
β-tocotrienol	0.19	**<0.001**	0.30	0.60	1.00	**<0.001**	**0.02**	1.00
γ-tocotrienol	0.28	**<0.001**	**0.03**	1.00	1.00	**0.02**	0.66	1.00
δ-tocotrienol	0.13	**<0.001**	**<0.01**	1.00	1.00	**<0.001**	**0.02**	1.00
total tocochromanols	0.23	**<0.001**	0.08	1.00	1.00	0.16	1.00	1.00

[a]Statistical significance stated at $p < 0.05$. [b]Tested as fixed effect, Wald-*F*-test. [c]Tested as random effect, loglikelihood ratio test.

physiological maturity is reached.[50] Therefore, it is likely that the plants already have compensated the differences in plant-available phosphate in the soil, explaining the lack of effect of phosphate fertilization.

Locational Influences. Although total tocochromanol concentrations of grains numerically increased in the order of Dettingen (69.5 μg/g DW), Eckartsweier (73.4 μg/g DW), and Hohenheim (74.0 μg/g DW), the concentration differences were not significant (Table 6). The concentrations of γ- and δ-tocopherol were significantly higher in grains from Hohenheim compared to Dettingen. These findings were partly in agreement with literature data about grains from corn grown at South Amana, Huxley, and Cambridge (United States), where a location effect was observed for γ-tocopherol ($p < 0.05$), but not for α-tocopherol.[42] In sunflower grains, a curvilinear relationship has been described between water supply and total tocopherol concentrations.[51] In the present study, the annual precipitation was higher in Hohenheim (856.5 mm) than in Eckartsweier (782.8 mm) or in Dettingen (661.4 mm). Thus, the significantly higher concentration of γ- and δ-tocopherol in the grains from plants grown in Hohenheim in comparison to grains from Dettingen might be due to higher precipitation without considering the water holding capacity of the soils. Further efforts should be made to verify this hypothesis under controlled conditions. Furthermore, low temperature stress has been described to lead to an accumulation of tocopherols in corn to protect polyunsaturated fatty acids,[14] which was not confirmed in the present study. In the case of tocotrienols, δ-tocotrienol concentrations were significantly lower in grains from Dettingen (0.9 μg/g DW) in comparison to Eckartsweier (1.1 μg/g DW). In corn seeds, an up to 6-fold accumulation of tocotrienols and tocopherols was triggered by overexpression of homogentisic acid geranylgeranyl transferase.[52] In this regard, a higher variation in the concentrations of tocopherols and tocotrienols may be achieved by transgenic approaches rather than by the choice of the location, even though the location in our experiment had an impact on the concentration of these compounds. Regarding the dietary allowances for vitamin E, 12 mg/day of α-tocopherol equivalents for female and 14 mg/day for male adults aged 25−51 years are recommended by the German Nutrition Society.[53] Thus, total tocopherols converted to α-tocopherol equivalents in 100 g of ground corn grains from Hohenheim would cover 18.0% of the recommended dietary allowances of vitamin E for female adults and 15.4% for male adults (aged 25−51 years). Total tocopherols in 100 g of ground grain samples from Dettingen would contribute 16.0% of the recommended dietary allowances for female and 13.7% for male adults, respectively. If only α-tocopherol concentrations were taken into consideration for the calculation of the adequate intake of vitamin E, as described by the European Food Safety Authority,[54] 9.1% (men) to 10.7% (women) of vitamin E would be covered by 100 g of ground corn from Hohenheim and 8.0% (men) to 9.5% (women) by 100 g of ground corn from Dettingen. Thus, the differences between the grain samples from different locations regarding their contribution to covering the recommended daily intake of vitamin E are negligible.

Concentration Differences between Corn Varieties. The concentrations of total tocochromanols and individual tocopherol and tocotrienol isomers were strongly affected ($p < 0.001$) by the variety (Table 5). As illustrated in Figure 3, tocochromanols accumulated especially in the grains of the varieties LG 30.258 (94.9 μg/g DW), Ricardinio (82.2 μg/g DW), and SY Talisman (76.9 μg/g DW). Significantly lower

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Table 6. Tocochromanol Concentrations of Grains from Corn Plants Grown at the Three Locations Hohenheim, Eckartsweier, and Dettingen in Germany

	concentration (μg/g DW)[a]		
tocochromanol	Hohenheim	Eckartsweier	Dettingen
α-tocopherol[b]	11.8a (10.2−14.5)	11.7a (8.5−13.2)	10.4a (8.2−14.0)
β-tocopherol[b]	1.0a (0.9−1.5)	1.0a (0.8−1.4)	0.9a (0.8−1.3)
γ-tocopherol[b]	37.1a (34.5−45.6)	32.7ab (28.8−41.9)	33.4b (29.1−39.8)
δ-tocopherol[b]	2.5a (2.1−3.0)	2.4ab (2.2−2.5)	2.0b (1.8−2.5)
α-tocotrienol[b]	8.8a (7.3−10.3)	8.0a (6.4−9.6)	8.7a (7.0−10.5)
β-tocotrienol[b]	0.9a (0.8−1.1)	0.9a (0.7−1.1)	0.8a (0.7−1.1)
γ-tocotrienol[b]	9.3a (5.7−12.5)	9.6a (7.0−14.9)	7.7a (4.9−12.7)
δ-tocotrienol[b]	1.0ab (0.8−1.2)	1.1a (0.9−1.5)	0.9b (0.6−1.0)
total tocochromanols[b]	74.0a (64.3−88.5)	73.4a (60.4−82.5)	69.5a (58.9−73.8)

[a]DW, dry weight. Concentration in the grains of eight corn varieties grown each in four plots was represented as median (interquartile range). Concentrations displaying different letters (a, b) show significant differences between the locations at $p < 0.05$. [b]Analyzed by pairwise Kruskal−Wallis test.

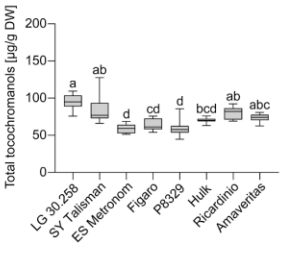

Figure 3. Boxplots of total tocochromanols in the grains of eight corn varieties. Medians were calculated based on the concentrations in the grains of each variety grown at the three sites in four plots. Different letters represent significant ($p < 0.05$) differences between medians.

concentrations were observed in the grains from Figaro (61.4 μg/g DW), ES Metronom (59.4 μg/g DW), and P8329 (57.9 μg/g DW). Thus, the total tocochromanols in the analyzed corn samples were higher than the average concentrations reported for spring wheat (34.5 μg/g DW), barley (31.5 μg/g DW), or winter wheat (24.5 μg/g DW).[55] A high heritability of total tocochromanols with 0.81 was estimated in a previous study investigating 20 genes in the grains of sweet corn for their association with total vitamin E content. The genes *hggt1* and *vte1* encoding for homogentisate geranylgeranyl transferase and tocopherol cyclase, respectively, were proposed as candidate genes for the large variation in tocotrienols,[9] which consequently impact the concentration of total tocochromanols. In our study, interaction effects between the variety and the location were significant for α-, β-, and δ-tocopherols and tocotrienols, but not for total tocochromanols and γ-tocopherol. α-tocopherol, being the vitamin E congener with the highest biological activity, is of great importance in human nutrition as an essential micronutrient.[56] Apparently, the concentration of total tocochromanols and α-tocopherol in corn on the field can be more controlled by the choice of the variety than by phosphate fertilization or the location within the temperate region.

In summary, the fatty acid, carotenoid, and tocochromanol profiles and concentration in the grains of modern corn hybrids appear to be unaffected by phosphate fertilization, at least when soils have met the recommendations in plant-available phosphate for optimum crop yields according to the Association of German Agricultural Analytic and Research Institutes (VDLUFA).[57] Regional differences appear to significantly affect the concentration of individual fatty acids, carotenoids, and tocochromanols. The corn variety, however, had a strong effect on total saturated and unsaturated fatty acids, total provitamin A carotenoids, total carotenoids, and total tocochromanols in the grains. This effect was complemented by significant interaction effects between the location and the variety on fatty acid proportions, total carotenoids, and total tocochromanols. Considering all of these findings, future breeding programs toward multiadaptive corn plants should aim to balance the effects of interregional climatic differences and soil nutrient-availability on grain yield and quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c07571.

Soil characteristics, coordinates, meteorological data, and growing periods of the three locations (Table S1); intraday and interday repeatabilities of the extraction methods for fatty acid methyl esters, carotenoids, and tocochromanols (Table S2); fatty acid composition in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany (Table S3); carotenoid concentration in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany (Table S4); tocochromanol concentration in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany (Table S5); chromatogram of (a) fatty acid methyl esters in ground corn grains after transesterification and (b) a standard mix containing 10 saturated or unsaturated fatty acid methyl esters (C_{14} to C_{22}) and ethyl myristate as internal standard; 1: methyl myristate; 2: ethyl myristate; 3: methyl palmitate; 4: methyl stearate; 5: methyl elaidate; 6: methyl oleate; 7: methyl linolelaidate; 8: methyl linoleate; 9: methyl linolenate; 10: methyl arachidate; 11: methyl behenate (Figure S1); chromatogram of carotenoids in ground corn grain extracts recorded at 450 nm; carotenoid standard mix including *β-apo*-8′-carotenal-methyloxime as internal standard was displayed dashed; 1: lutein; 2: zeaxanthin; 3: β-cryptoxanthin; 4: *β-apo*-8′-carotenal-methyloxime; 5: α-carotene; 6: β-carotene

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(Figure S2); chromatogram of tocopherols and tocotrienols in ground corn grains (excitation wavelength, 296 nm; emission wavelength, 325 nm); chromatogram of authentic tocopherol and tocotrienol standards is displayed in grayscale; 1: δ-tocotrienol; 2: β-tocotrienol; 3: γ-tocotrienol; 4: α-tocotrienol; 5: δ-tocopherol; 6: β-tocopherol; 7: γ-tocopherol; 8: α-tocopherol (Figure S3) (PDF)

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Notes

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ABBREVIATIONS

ANOVA, analysis of variance; BHT, butylated hydroxytoluene; DW, dry weight; EI, electron ionization; FAME, fatty acid methyl esters; FID, flame ionization detector; FLD, fluorescence detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; P, phosphorus; UV/vis, ultraviolet/visible light detection; VDLUFA, Association of German Agricultural Analytic and Research Institutes

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Supporting Information

Location and Variety but Not Phosphate Starter Fertilization Influence the Profiles of Fatty Acids, Carotenoids, and Tocochromanols in Kernels of Modern Corn (*Zea mays* L.) Hybrids Cultivated in Germany

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Table S1. Soil characteristics, coordinates, meteorological data, and growing periods of the three locations

	Hohenheim	Eckartsweier	Dettingen
Coordinates	Latitude 48°43'05.7"N, longitude 9°11'20.8"E	Latitude 48°32'24.7"N, longitude 7°51'15.1"E	Latitude 48°35'32.3"N, longitude 10°08'23.4"E
Altitude	409 m	136 m	563 m
Soil texture	Silty loam	Clayey loam	Clayey loam
Soil pH [a]	6.96	6.68	7.20
Soil P concentration [b] [mg CAL-P/100 g soil]	8.5	8.4	20.6
Annual mean temperature [cd] [°C]	10.6	11.7	9.1
Annual precipitation [cd] [mm]	856.5	782.8	661.4
Sum of solar irradiance [cd] [kWh/m²]	1239	1220	864
Sowing date	April 29, 2019	April 23, 2019	May 6, 2019
Harvesting date	October 15-16, 2019	September 24, 2019	October 29, 2019

[a] Determined in $CaCl_2$ solution. [b] Extracted by using the calcium-acetate-lactate (CAL-P) method. [c] In the cultivation year 2019. [d] Data were acquired from weather stations in the vicinity of the locations (www.wetter-bw.de/Agrarmeteorologie-BW/Wetterdaten/Stationskarte)

Table S2. Intraday and interday repeatabilities of the extraction methods for fatty acid methyl esters, carotenoids, and tocochromanols

	Intraday repeatability [a] [CV%]	Interday repeatability [b] [CV%]
Total saturated fatty acids [c]	0.56	0.18
Total unsaturated fatty acids [c]	0.10	0.03
Total provitamin A carotenoids	2.83	6.88
Total carotenoids	3.27	8.66
Total tocochromanols	5.13	9.69

CV, coefficient of variation. [a] Mean value of six measurements within one day. [b] Mean value of six measurements from three days within three weeks. [c] Determined as fatty acid methyl esters.

Table S3. Fatty acid composition in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany

Fatty acid	Fatty acid composition [%] [a]	
	+P	P0
Palmitic acid (16:0)	12.8 (12.2−13.7)	12.9 (12.3−13.6)
Stearic acid (18:0)	1.9 ± 0.2	1.8 ± 0.2
Arachidic acid (20:0)	0.9 (0.9−1.0)	0.9 (0.8−1.1)
Oleic acid (18:1*n*-9)	26.0 (24.6−27.6)	26.0 (24.6−27.9)
Linoleic acid (18:2*n*-6)	57.6 ± 2.9	57.6 ± 3.1
α-Linolenic acid (18:3*n*-3)	0.7 (0.6−0.7)	0.7 (0.6−0.7)
Total saturated fatty acids	15.7 (15.1−16.6)	15.6 (15.0−16.6)
Total unsaturated fatty acids	84.3 (83.4−84.9)	84.4 (83.4−85.0)

+P, cultivated with 52.9 kg P ha^{-1}. *P0*, cultivated without phosphate fertilizer. Composition in the grains of eight corn varieties grown at three sites and in replicated plots was represented as mean ± standard deviation or median (interquartile range). [a] Expressed as percentage of fatty acid methyl esters.

Table S4. Carotenoid concentration in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany

Carotenoid	Concentration [µg/g DW]	
	+P	P0
Lutein	20.1 (17.0−25.2)	20.0 (17.2−25.5)
Zeaxanthin	12.4 (10.4−14.2)	12.1 (10.2−14.3)
β-Cryptoxanthin	1.8 (1.3−2.4)	1.8 (1.3−2.2)
α-Carotene	6.8 (0.5−0.9)	6.8 (0.5−0.9)
β-Carotene	2.1 (1.7−2.8)	2.0 (1.7−2.9)
Total provitamin A carotenoids	4.6 (4.1−5.5)	4.7 (4.1−5.5)
Total carotenoids	36.5 (33.6−41.6)	35.3 (32.9−41.7)

DW, dry weight. *+P*, cultivated with 52.9 kg P ha^{-1}. *P0*, cultivated without phosphate fertilizer. Concentration in the grains of eight corn varieties grown at three sites and in replicated plots was represented as median (interquartile range).

Table S5. Tocochromanol concentration in corn grains of eight hybrids grown with or without phosphate fertilizer at the three locations Hohenheim, Eckartsweier, and Dettingen in Germany

Tocochromanol	Concentration [µg/g DW]	
	+P	P0
α-Tocopherol	11.8 (8.3−13.6)	10.7 (8.3−13.2)
β-Tocopherol	1.0 (0.8−1.4)	0.9 (0.9−1.3)
γ-Tocopherol	37.0 (42.2−30.5)	34.8 (42.6−30.2)
δ-Tocopherol	2.3 (2.0−2.6)	2.3 (2.0−2.7)
α-Tocotrienol	8.7 (7.1−10.3)	8.2 (7.0−10.0)
β-Tocotrienol	0.9 (0.7−1.1)	0.8 (0.7−1.1)
γ-Tocotrienol	9.4 (5.9−12.8)	9.0 (5.7−11.8)
δ-Tocotrienol	1.0 (0.9−1.3)	0.9 (0.8−1.2)
Total tocochromanols	73.0 (62.8−81.7)	70.0 (60.4−81.3)

DW, dry weight. *+P*, cultivated with 52.9 kg P ha^{-1}. *P0*, cultivated without phosphate fertilizer. Concentration in the grains of eight corn varieties grown at three sites and in replicated plots was represented as median (interquartile range).

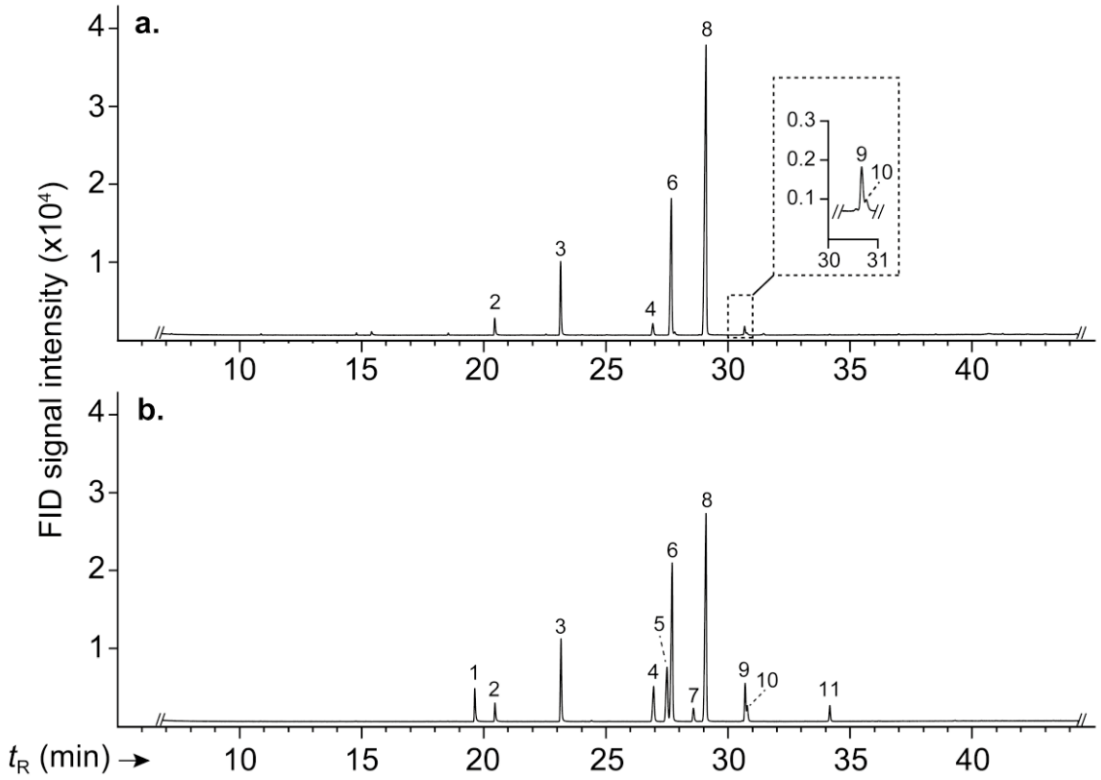

Figure S1. Chromatogram of (a) fatty acid methyl esters in ground corn grains after transesterification and (b) a standard mix containing 10 saturated or unsaturated fatty acid methyl esters (C_{14} to C_{22}) and ethyl myristate as internal standard. 1: Methyl myristate; 2: Ethyl myristate; 3: Methyl palmitate; 4: Methyl stearate; 5: Methyl elaidate; 6: Methyl oleate; 7: Methyl linolelaidate; 8: Methyl linoleate; 9: Methyl linolenate; 10: Methyl arachidate; 11: Methyl behenate.

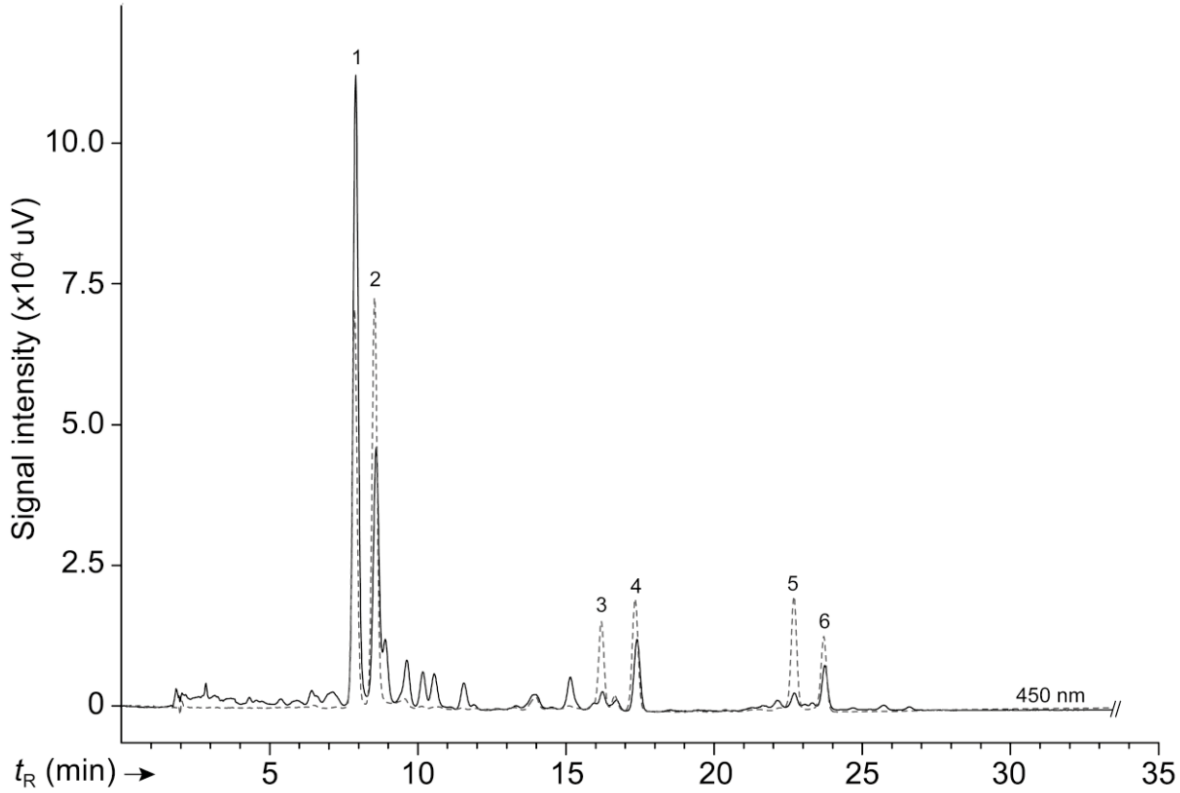

Figure S2. Chromatogram of carotenoids in ground corn grain extracts recorded at 450 nm. The carotenoid standard mix including β-*apo*-8′-carotenal-methyloxime as internal standard was displayed dashed. 1: Lutein; 2: Zeaxanthin; 3: β-Cryptoxanthin; 4: β-*apo*-8′-Carotenal-methyloxime; 5: α-Carotene; 6: β-Carotene.

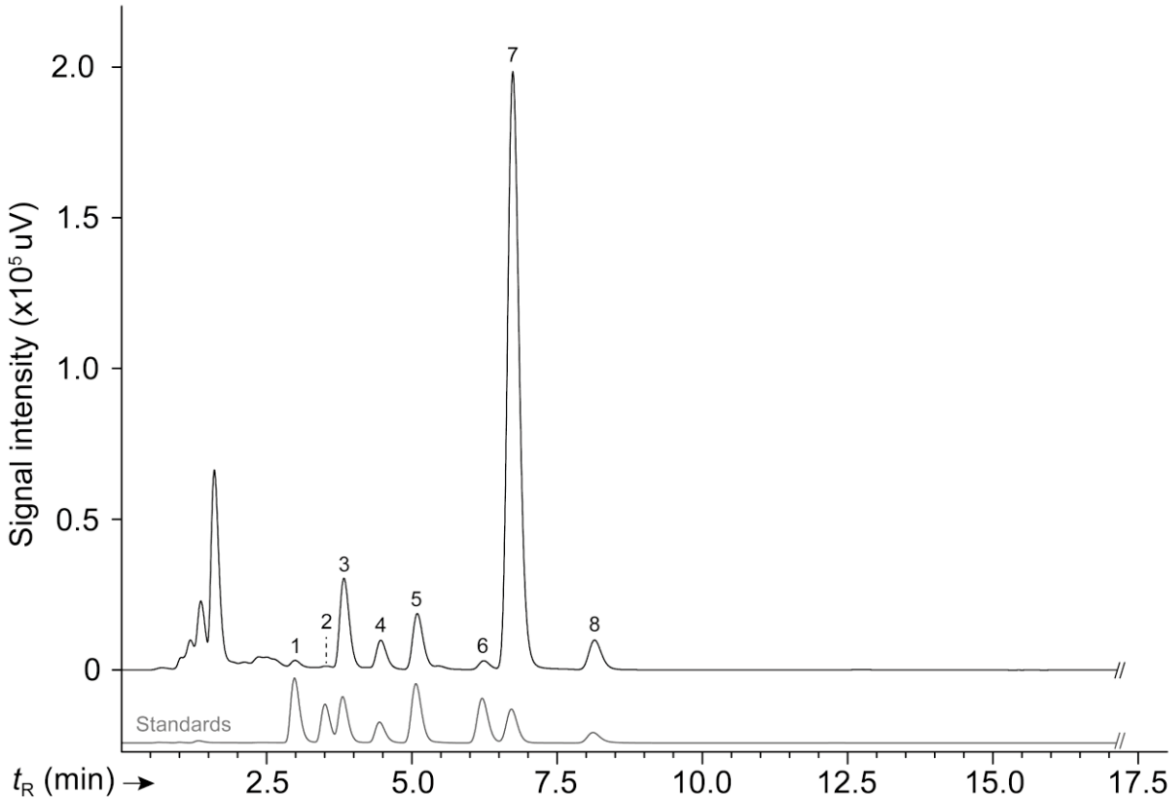

Figure S3. Chromatogram of tocopherols and tocotrienols in ground corn grains (excitation wavelength 296 nm; emission wavelength, 325 nm). Chromatogram of authentic tocopherol and tocotrienol standards is displayed in grayscale. 1: δ-Tocotrienol; 2: β-Tocotrienol; 3: γ-Tocotrienol; 4: α-Tocotrienol; 5: δ-Tocopherol; 6: β-Tocopherol; 7: γ-Tocopherol; 8: α-Tocopherol.

Chapter 4

Oxidative stability of tocochromanols, carotenoids, and fatty acids in maize (*Zea mays* L.) porridges with varying phytate concentrations during cooking and in vitro digestion

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Corrigendum to "Oxidative stability of tocochromanols, carotenoids, and fatty acids in maize (*Zea mays* L.) porridges with varying phytate concentrations during cooking and in vitro digestion" [Food Chem. 378 (2022) 132053]

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The authors regret a mistake in the abstract that describes the effect of phytic acid on the micellarization efficiencies of carotenoids as the opposite of the effect actually observed. The erroneous statement in the abstract <The addition of phytic acid did not affect the digestive stabilities of total tocochromanols and carotenoids, but increased micellarisation efficiencies of carotenoids> should correctly read <The addition of phytic acid did not affect the digestive stabilities of total tocochromanols and carotenoids, but **decreased** micellarisation efficiencies of carotenoids>.

The authors would like to apologise for any inconvenience caused.

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Oxidative stability of tocochromanols, carotenoids, and fatty acids in maize (*Zea mays* L.) porridges with varying phytate concentrations during cooking and in vitro digestion

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ABSTRACT

Phytic acid, the main storage form of phosphate in maize (*Zea mays* L.) grains, is known as antinutrient due to its chelating properties but may also prevent oxidation. Thus, the impact of phytic acid on the degradation of tocochromanols, carotenoids, fatty acids, and oxidation products in maize during cooking and subsequent in vitro digestion was examined. Maize porridges from low phytic acid maize flour with or without admixed phytate, or from high phytic acid maize flour were prepared, and digestion experiments conducted. HPLC-(MS) or GC-MS analyses revealed a significant decrease in tocochromanols, carotenoids, and unsaturated fatty acids in the digesta compared to the maize porridges while α-tocopherylquinone and malondialdehyde concentrations increased. The addition of phytic acid did not affect the digestive stabilities of total tocochromanols and carotenoids, but increased micellarisation efficiencies of carotenoids. In conclusion, phytate did not exert antioxidant effects in maize porridge during cooking or simulated digestion.

1. Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate), also known as the anion phytate, is a mineral chelator and the main storage form of phosphate in seeds (Kumar et al., 2010). Due to its high affinity for minerals, such as iron, phytate impairs their bioavailability for humans (Hurrell et al., 2003). Especially in developing countries, where maize, sorghum, and millets are consumed as complementary foods, the binding effect of phytate contributes to iron and zinc deficiencies (Gabaza et al., 2017). Phytate has also been suggested to decrease iron-induced hydroxyl radical formation and to suppress lipid peroxidation due to its high binding affinity for iron (Kumar et al., 2010). Moreover, a maize mutant with defective phytate synthesis was reported to have higher contents of free and weakly bound iron and a higher production of free radicals compared to wild-type maize (Doria et al., 2009). It should also be noted that phytate concentrations in maize grains show a remarkable variation between genotypes (Rodehutscord et al., 2016; Sun et al., 2022), and can be increased by phosphorus application (Modi & Asanzi, 2008). Maize also contains carotenoids and tocochromanols (tocopherols and tocotrienols), which are potent antioxidants (Anguelova & Warthesen, 2000; Lux et al., 2021; Alberdi-Cedeño et al., 2019). Characteristic products are formed when macromolecules are oxidized, such as malondialdehyde from polyunsaturated fatty acids and tocopherylquinones from tocopherols (Doria et al., 2009; Liebler & Burr, 1992).

However, the effects of phytate contents of maize on the oxidation of lipids and the formation of oxidation endproducts during processing and digestion have not been investigated. Maize porridge was chosen as test meal, because it mainly is composed of water and ground maize grains, all fractions of the maize grain are included, and it represents a widely consumed dish in African countries, where phytic acid-induced impaired absorption of minerals and resulting deficiencies are common (Hurrell et al., 2003). Thus, we hypothesized that the oxidative stability of tocochromanols, carotenoids, and the fatty acid profile of maize porridge is influenced by the presence of phytic acid during cooking and

Abbreviations: ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionisation; BHT, butylated hydroxytoluene; DAD, diode array detector; DW, dry weight; FAME, fatty acid methyl esters; FLD, fluorescence detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; ICP-OES, inductively coupled plasma-optical emission spectrometry; $MS^{(n)}$, (multi-stage) mass spectrometry; $NaHCO_3$, sodium hydrogen carbonate; SIM, selected ion monitoring; UV/Vis, ultra-violet/visible light.

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digestion and tested this experimentally.

2. Material and methods

2.1. Chemicals

L(+)-Ascorbic acid (purity ≥ 99%), acetone (purity ≥ 99.5%), butylated hydroxytoluol (BHT), ethanol, glacial acetic acid, nitric acid, *n*-hexane, potassium dihydrogen phosphate (purity ≥ 98%), potassium hydroxide solution (50%, *w/v*), sodium chloride (purity ≥ 99%), sodium acetate trihydrate (purity ≥ 99.5%), sodium hydrogen carbonate ($NaHCO_3$, purity ≥ 99.5%), sulfuric acid (purity ≥ 95%), and trichloroacetic acid (purity ≥ 99%) were obtained from Carl-Roth (Karlsruhe, Germany). α-Amylase from *Aspergillus oryzae* (35.9 U/mg), α-carotene, bile extract porcine, diethyl ether (≥ 99%, inhibitor-free), ethyl myristate, analytical standard mix comprising saturated and unsaturated fatty acid methyl esters (C_{14} to C_{22}), hydrochloric acid (37%, *w/w*), lipase from porcine pancreas (388 U/mg), pancreatin from porcine pancreas (8 × USP specifications), pepsin (599 U/mg), α–, β-, γ–, δ–tocopherol and -tocotrienol standards (purity ≥ 95.5%), and α-tocopherylquinone were purchased from Sigma-Aldrich (Taufkirchen/Steinheim, Germany). *Myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate (> 95% purity) was obtained from SiChem (Bremen, Germany). Multi-element calibration standards including zinc and iron were from Inorganic Ventures (Christiansburg, VA, USA). Thiobarbituric acid was obtained from AppliChem (Darmstadt, Germany). Ammonium heptamolybdate was purchased from Merck (Darmstadt, Germany). Methanol was from J.T. Baker (Gliwice, Poland). Lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were purchased from Extrasynthèse (Genay, France). Ultra-purified water was prepared by a Milli-Q (Millipore, Billerica, MA, USA) system. All solvents were of high purity (HPLC grade).

2.2. Plant materials and porridge preparation

Maize genotypes with a low (10.9 μmol/g dry weight, DW) and high (16.6 μmol/g DW) phytic acid concentration were selected from a panel comprising 27 maize genotypes. An extensive chemical characterisation, including phytic acid (ranging from 1.94 to 3.09 g/kg DW, mean 2.26 g/kg DW), of the selected genotypes was published previously (Rodehutscord et al., 2016). Maize grains were pre-milled (Rotor GT 800, Rotor AG, Uetendorf, Switzerland) and ground to a particle size of ≤ 500 μm (Fritsch Pulverisette 14, Frisch, Idar-Oberstein, Germany). The term flour was used in the article describing the ground maize grains.

Maize porridges were prepared based on a previously published protocol (Faber et al., 2005). For each porridge, 20 g of finely ground low or high phytic acid maize were resuspended in 125 mL of distilled water. An additional batch of porridge was prepared with 20 g of ground low phytic acid maize flour and a sodium phytate solution in order to reach the same phytic acid concentration as the high phytic acid maize. The suspensions were heated under agitation (90 °C, 5 min). The resulting porridges were immediately cooled on ice and transferred into resealable containers. Porridges and ground maize samples were stored at −80 °C under nitrogen atmosphere until analysis.

2.3. In vitro digestion experiments

Three-stage in vitro digestion was performed according to Lipkie et al. (2013) with minor modifications. The oral phase was initiated by mixing 8 g of porridge with 6 mL of α-amylase solution (3000 units). The sample was vortexed, headspace flushed with nitrogen, and horizontally placed into a light-protected shaking water bath (180 rpm, 37 °C, 2 min). The incubated sample was immediately cooled on ice. For the gastric phase, pepsin (target concentration, 0.5 mg/mL) and sodium chloride (0.9%, *w/v*) solution were added. The pH was adjusted to 2.5 ± 0.1 with 1 M hydrochloric acid. The mixture was vortexed, purged with nitrogen, and incubated horizontally in the water-bath (180 rpm, 37 °C, 1 h). The sample was placed on ice. For the intestinal phase, bile extract (target concentration 1.8 mg/mL), pancreatin (0.8 mg/mL), and lipase (0.4 mg/mL), dissolved each in 100 mM $NaHCO_3$ solution, were added. The pH was checked and adjusted to 6.5 ± 0.1 with $NaHCO_3$ solution, as appropriate. The sample was gently vortexed and incubated under nitrogen atmosphere in the water bath (180 rpm, 37 °C, 2 h). The digested sample was put on ice. A subsample of digesta was transferred into a centrifuge tube, headspace-flushed with nitrogen, and stored at −80 °C until analysis. The remainder was centrifuged (10,000 × *g*, 4 °C, 1 h). An aliquot of the supernatant (aqueous phase) was transferred into a centrifuge tube, purged with nitrogen, and frozen at −80 °C. The remainder was sterile filtrated (0.2 μm pore size), and the filtrate (micellar phase) was stored at −80 °C under nitrogen atmosphere. Maize porridge and digesta were lyophilized in light-protected flasks prior to extraction and analyses of phytate, minerals, fatty acids, and malondialdehyde. Dry substance of ground maize grains, digesta, and porridge was determined gravimetrically in a drying oven.

2.4. Analyses of phytic acid, minerals, tocochromanols, carotenoids, fatty acids and oxidation products

2.4.1. Extraction and analyses of phytic acid and minor inositolphosphates by ion chromatography

Inositolphosphates were extracted and analysed according to Zeller et al. (2015). In brief, 1 g of sample (maize flour, freeze-dried maize porridge or digesta) was mixed with 0.2 M ethylenediaminetetraacetic acid and 0.1 M sodium fluoride solution. The suspension was centrifuged (12,000 × *g*, 6 °C, 15 min) and the supernatant collected in another centrifuge tube and cooled on ice. The remainder was re-extracted with ethylenediaminetetraacetic acid and sodium fluoride solution. The supernatants were combined, and a subsample of 1 mL was centrifuged (14,000 × *g*, 6 °C, 15 min). The liquid phase was filtered (0.2 μm pore size, cellulose acetate) and the filtrate centrifuged (14,000 × *g*, 6 °C) in a Microcon filter for 30 min. The final filtrates were injected into an ICS-3000 ion chromatography system (Dionex) with post-column derivatization. Inositolphosphates were detected at a wavelength of 290 nm after derivatization.

2.4.2. Analyses of minerals by ICP-OES

Transition metals (zinc, iron) in maize flour, freeze-dried porridge, or digesta were extracted using microwave-assisted acid digestion and were analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) according to the official methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2011, 1995).

2.4.3. Extraction and analyses of tocochromanols by HPLC-FLD

Aliquots of maize flour (50 mg), porridge (500 mg), digesta, aqueous or micellar phases (2 mL each) were extracted with *n*-hexane, and the organic solvent was evaporated under reduced pressure as described previously (Grebenstein & Frank, 2012; Lux et al., 2020). In order to achieve a clear phase separation after mixing the sample with *n*-hexane, centrifugal forces and centrifugation time were adjusted (3,000 × *g*, 4 °C, 10 min). The obtained extracts were dissolved in ethanol and analysed by high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) using the following conditions: A Shimadzu (Kyoto, Japan) Prominence HPLC, equipped with a Kinetex pentafluorophenyl column (100 × 4.6 mm i.d., 2.6 μm particle size; Phenomenex, Aschaffenburg, Germany), was used. A sample volume of 10 μL was injected and the flow rate was 0.6 mL/min. Eluent A consisted of methanol/water (80/20, *v/v*) and eluent B was methanol/water (97/3, *v/v*). Gradient elution was applied starting with 0% B to 100% B (20 min), hold at 100% B (5 min), flushed back to 0% B (2 min) and isocratically hold (3 min) under these conditions (Montoya-Arroyo et al., 2021). The column oven temperature was held at 40 °C. FLD was used

with an excitation wavelength of 296 nm and an emission wavelength of 325 nm. External standard curves of α-, γ-, and δ-tocopherols and -tocotrienols were created for quantitation. Concentrations of individual tocopherols and tocotrienols were summarised as total tocochromanol concentration. Digestive stabilities were calculated based on Werner and Böhm (2011) as the percent ratio of analyte concentration in the digesta to the analyte concentration in the porridge. Solubility (percent ratio of the analyte concentration in the aqueous fraction to the analyte concentration in the porridge) and micellarisation efficiency (percent ratio of the analyte in the micellar fraction to the analyte concentration in the porridge) were calculated.

2.4.4. Extraction and analyses of α-tocopherylquinone by HPLC-DAD-APCI-MS^n

Maize flour (100 mg), maize porridge (1 g), digesta (3 mL) were each weighed into a centrifuge tube. The sample was diluted with 1 mL of water and 1 mL of ethanol. The suspension was mixed with 2 mL of hexane/diethyl ether (50/50, *v/v*) and centrifuged (1,687 × *g*, 4 °C, 3 min). The organic supernatant was transferred into another centrifuge tube. The extraction was repeated one more time with 2 mL of the extracting agent. The combined organic layers were immediately evaporated under reduced pressure. The obtained extracts were dissolved in ethanol and filled into amber glass vials for HPLC-MS analysis. Clean glassware was used throughout the extraction process.

The samples were analysed by HPLC-MS using an Agilent 1290 series HPLC connected to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The same chromatographic settings as described in section 2.4.3 were applied. UV/Vis spectra were recorded in the range of 190 to 600 nm. Mass spectra were acquired using atmospheric pressure chemical ionisation (APCI) in positive mode at a scan range of *m/z* 100 to 1000. The scan range for the fragmentation experiments was chosen automatically. The retention time, UV absorption maximum and fragmentation pattern of α-tocopherylquinone was confirmed by an authentic standard (Supplementary data, Fig. S1). The peak area of the predominant protonated molecular ion after in-source fragmentation of water $[M + H\text{-}H_2O]^+$ at *m/z* 429 was selected for quantitation.

Recovery was determined in triplicate with an added α-tocopherylquinone standard (5 μM) resulting in a mean recovery of 96% ± 6%. Intra-day repeatability (coefficient of variation, 10%; six determinations) and linearity within the calibration range of 1 to 75 μM (correlation coefficient, 0.992; slope of the regression line, 5.8×10^9; y-intercept, 1.9×10^9; residual sum of squares, 1.4×10^{20}, six calibration points) of α-tocopherylquinone were assessed considering the guidelines of the International Council for Harmonisation (ICH Expert Working Group, 2005).

2.4.5. Extraction and analyses of carotenoids by HPLC-UV/Vis

Carotenoids were extracted and analysed by HPLC-UV/Vis according to the method of Lux et al. (2020). Briefly, flour (100 mg), porridge (1 g), digesta, aqueous or micellar phase (2 mL each) were saponified in an ethanolic potassium hydroxide solution in the presence of BHT and internal standard. The sample was cooled on ice, sodium chloride solution (15%, *w/v*) was added and neutralized with glacial acetic acid. Solvent extraction with *n*-hexane/diethyl ether (50/50, *v/v*) and two extraction cycles was performed. The organic phases were pooled and evaporated to dryness under reduced pressure. The obtained extracts were dissolved in pure ethanol and analysed by a Shimadzu Prominence HPLC-UV/Vis. Analytes were separated on a C30 Develosil RP-aqueous column (250 × 4.6 mm i.d., 5.0 μm particle size; Phenomenex) using gradient elution. Chromatograms were recorded at a wavelength of 450 nm. Individual carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α-, and β-carotene) were quantitated and summed up as total carotenoids. Digestive stabilities, solubilities, and micellarisation efficiencies were calculated for the individual and total carotenoids as described in section 2.4.3.

2.4.6. Extraction and analyses of fatty acids as fatty acid methyl esters (FAME) by GC-MS

Fatty acids in maize flour, freeze-dried porridge or digesta (15 mg each) were transesterified with sulfuric methanol (1%, *w/v*) in a covered waterbath (80 °C, 4 h) as published by Lux et al. (2021). The resulting fatty acid methyl esters were extracted with *n*-hexane. An aliquot of the organic phase was combined with ethyl myristate as internal standard (0.2 mg/mL) and transferred into an amber glass vial. FAME were analysed by a 5890 Series II Plus gas chromatograph connected with a 5972 Series mass detector (both from Hewlett-Packard/Agilent, Waldbronn, Germany). An Rtx-2330 column (60 m × 0.25 mm i.d., 0.1 μm film thickness; Restek, Bellefonte, PA) with biscyanopropyl (90%) and phenylcyanopropyl siloxane (10%) coating was used. A volume of 1 μL was injected by a 7673 autosampler (Hewlett-Packard/Agilent). The injector temperature was kept at 250 °C. The transfer line temperature was 270 °C. Helium (purity 99.999%) was used as carrier gas. The gas flow rate was 1 mL/min. The temperature programme was detailed in a previous study (Lux et al., 2021). Full scan (*m/z* 50 to 550) and selected ion monitoring (SIM, *m/z* 74, 79, 81, 87, 88, and 101) mode were applied (Wiedmaier-Czerny et al., 2021). Percentage distribution of FAME was calculated based on the peak areas of the selected ions (Thurnhofer & Vetter, 2005).

2.4.7. Analyses of malondialdehyde equivalents by spectrophotometry

Concentration of malondialdehyde equivalents in flour, freeze-dried porridge or digesta (200 mg each) was spectrophotometrically assessed at 532 nm after condensation with thiobarbituric acid using the protocol of Doria et al. (2009).

2.5. Determination of phytase activity

The assay for the determination of phytase activity in the enzyme extracts was based on the method of Nuobariene et al. (2011). Briefly, enzyme extracts used for in vitro digestion or bile salts were dissolved in 0.2 M sodium acetate buffer (pH 5.5). The solution was centrifuged (5,000 × *g*, 4 °C, 20 min). Phytic acid solution (3 mM), dissolved in acetate buffer (pH 5.5), was preincubated in a thermocycler at 30 °C. Enzyme solution (10 mg/mL) was added, slightly vortexed, and incubated in a thermocycler (800 rpm, 30 °C). Trichloroacetic acid was added to stop the hydrolysis after an incubation time of 45 min. A control sample was prepared by adding trichloroacetic acid prior to the addition of the enzyme solution. An aliquot was taken and mixed with acidic ammonium molybdate solution (2.5 mM). The amount of released inorganic phosphate was determined photometrically at 355 nm. A standard curve was prepared from a potassium dihydrogen phosphate stock solution (2 mM) and the absorption measured photometrically. Phytase activity was expressed as unit (U) which defines the amount of enzyme that releases 1 μmol per mL of inorganic phosphate per min from a 3 mM phytic acid solution at pH 5.5 and 30 °C.

2.6. Statistical analyses

Six independent digestion experiments were performed for each group (low phytic acid maize porridge, low phytic acid maize porridge with phytate, high phytic acid maize porridge). Results were expressed as mean ± standard deviation. *T*-test (two data sets) or one-way analysis of variance (ANOVA, three data sets) with Bonferroni post-hoc test were conducted assuming Gaussian normal distribution and homogeneity of variances. Statistical analyses were performed with SPSS statistics version 25 (IBM, Armonk, NY, USA). Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Quantitation of phytic acid, zinc, and iron in maize after cooking and in vitro digestion

The mean concentrations of phytic acid dropped from initially 10.9 μmol/g of dry weight (DW) in the low phytic acid maize flour and 16.6 μmol/g of DW in the high phytic acid maize flour to 10.1 μmol/g of DW and 14.4 μmol/g of DW, respectively, after cooking (Fig. 1). The reduction in the phytic acid concentration was accompanied by an increase in *myo*-inositol-1,2,4,5,6-penta*kis*phosphate to 0.2 μmol/g of DW for the low phytic acid maize sample and to 0.4 μmol/g of DW for the high phytic acid maize samples. *Myo*-inositol-1,2,3,4,5-penta*kis*phosphate was detected in traces (< 0.2 μmol/g of DW) in the low phytic acid maize samples after cooking. Mean concentration of *myo*-inositol-1,2,3,4,5-penta*kis*phosphate were not detected in the uncooked high phytic acid maize flour but its concentrations rose to 0.3 μmol/g of DW after cooking. A reduction in the phytate content by up to 14 ± 5.7% has been observed after soaking ground maize in water, and the effects on phytate reduction was more pronounced for milled grains than for whole grains (Kruger et al., 2014). An even stronger phytate reduction by up to 95% was achieved in wheat by combining germination with hydrothermal processing at pH 3.8 and a temperature of 50 °C for 24 h (Lemmens et al., 2018). In our study, the increase in minor phosphates with a concomitant decrease in phytic acid may indicate that phytic acid was dephosphorylated during the warm-up and cooking process. This observation could be explained by an enzymatic hydrolysis due to the weak intrinsic phytase activity of the used maize genotypes (Rodehutscord et al., 2016).

After in vitro digestion of the cooked maize flour (porridge), the concentration of phytic acid in the digesta were significantly lower resulting in 5.9 μmol/g of DW for the low phytic acid maize and 9.7 μmol/g of DW for the high phytic acid maize porridge. Even though phytase (*EC* 3.1.3.8) activity has been observed in mammals, for instance in mucosal homogenates of the small intestine of rats and guinea pigs (Cooper & Gowing, 1983), a degradation of phytic acid by about fifty percent after the small intestinal phase was unexpected. Thus, phytase activity of the applied enzyme and bile extracts was assayed. Interestingly, a phytase activity of 2.0 mU/mg was found in the α-amylase extract added at the first stage of in vitro digestion. The α-amylase was originally isolated from the mould *Aspergillus oryzae*. According to published data, *Aspergillus oryzae* was able to secret protease-resistant phytase (Sapna & Singh, 2014). For this reason, it is possible that phytic acid in the porridges was hydrolysed by phytase present in the α-amylase extract. Since the degradation rate of phytic acid in the low phytic acid maize samples with 58.6% was close to the degradation rate in the high phytic acid maize samples with 62.9% after in vitro digestion, the impact of phytic acid on tocochromanols, carotenoids, fatty acids, and oxidation products was still examined.

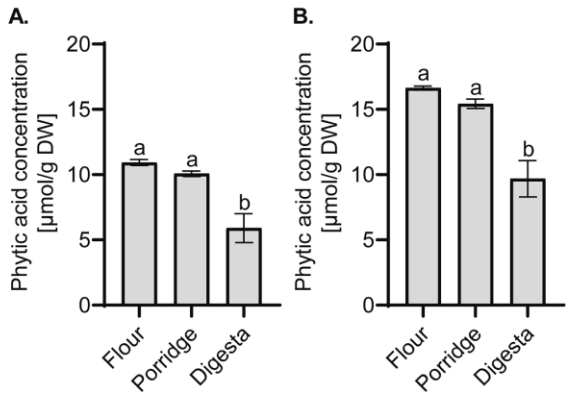

Fig. 1. Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate) concentrations in flour, porridge, and digested maize porridge of low phytic acid maize (A) and high phytic acid maize (B). *DW*, dry weight. Bars not sharing a lower-case letter are significantly different ($p < 0.05$).

Compared to the concentration of phytic acid, the zinc and iron concentrations in the maize samples were rather stable after cooking and digestion. The iron concentrations in the flour, porridge, and digesta ranged between 20.65 and 22.53 μg/g of DW for low phytic acid maize and between 25.22 and 26.35 μg/g of DW for high phytic acid maize (Supplementary data, Table S1). Flour, porridge and digesta exhibited zinc concentrations of 19.42 to 20.63 μg/g of DW for low phytic acid maize and 19.18 to 20.93 μg/g of DW for the high phytic acid maize samples.

3.2. Effect of phytate, cooking, and in vitro digestion on tocochromanols, carotenoids, fatty acids, and oxidation products in maize porridge

3.2.1. Tocochromanols

In the flour, porridge, and digesta of both genotypes used for this study, α-, γ- and δ-tocopherols and -tocotrienols were identified and quantified. The concentrations of total tocochromanols were initially lower in the flour of low phytic acid maize with 58.58 μg/g of DW than the concentration in the flour of high phytic acid maize with 150.93 μg/g of DW (Table 1). After the cooking process, the total tocochromanol concentration significantly ($p < 0.05$) decreased to 43.01 μg/g of DW in the low phytic acid maize sample and to 129.02 μg/g of DW in the high phytic acid maize sample. The total tocochromanol concentration in the low phytic acid maize porridge spiked with phytic acid was also reduced compared to the low phytic acid flour resulting in 52.82 μg/g of DW. For this reason, it appears that the addition of phytic acid has not fully prevented the degradation of tocochromanols in porridge during cooking. In relation to the low phytic acid flour, total tocochromanols degraded by 9.8% in the porridge of maize sample spiked with phytate and by 26.5% in the low phytic acid maize sample indicating to some extend a protective effect by phytic acid. However, the degradation rate of total tocochromanols in the high phytic acid maize with 14.5% was still higher than the degradation of the low phytic acid maize spiked with phytate. It should be noted that interaction of tocopherols with other radical-scavenging or tocopherol-regenerating compounds have been described in the literature (Kamal-Eldin & Appelqvist, 1996). This weakened the argument that solely phytic acid was causing protection against tocochromanol degradation during cooking of maize.

After in vitro digestion of the porridge, a significant decrease in the total tocochromanol concentration was observed for all three samples. The total tocochromanol concentrations in the final digesta were lowest in the low phytic acid maize sample with 13.23 μg/g of DW followed by the low phytic acid maize sample spiked with phytate with 17.29 μg/g of DW and the high phytic acid maize sample with 71.77 μg/g of DW. The decrease in total tocochromanols from porridge to digesta was primarily attributed to the significant degradation of γ- and α-tocopherol which were the most abundant tocochromanols in the maize sample material. The superior degradation of γ- compared to α-tocopherol was in agreement with the results in maize germ oil under accelerated oxidation conditions at 70 °C revealing degradation kinetics of tocopherols in the descending order of γ-, α-, δ- and β-tocopherols (Alberdi-Cedeño et al., 2019). Previous findings supported the potent antioxidative role of γ-tocopherol in γ-tocopherol enriched maize oil during in vitro digestion reducing the concentrations of volatile oxidation products such as individual alkanals, furan derivatives, and 2,4-alkadienals in the headspace of the digesta compared to digesta of pure corn oil (Alberdi-Cedeño et al., 2020). γ-Tocotrienol was the main tocotrienol in the digesta of low phytic acid maize porridge with 1.25 μg/g of DW, in the digesta of low phytic acid maize porridge spiked with phytate with 1.85 μg/g of DW and in the high phytic acid maize digesta with 7.03 μg/g of DW. Nevertheless, the addition of phytate did neither impede a

Table 1
Tocochromanol and carotenoid concentrations in flour, porridge, and digested maize porridge of low phytic acid maize (LM), low phytic acid maize supplemented with phytate (LMS), and high phytic acid maize (HM).

Compound	Sample material	Concentration [µg/g DW]		
		Flour	Porridge	Digesta
α-Tocopherol	LM	9.32 ± 1.50^{a}	6.46 ± 1.38^{b}	1.47 ± 0.32^{c}
	LMS	–	7.20 ± 3.04^{a}	1.46 ± 0.23^{b}
	HM	49.64 ± 5.12^{a}	43.94 ± 5.55^{a}	20.92 ± 2.17^{b}
γ-Tocopherol	LM	37.43 ± 3.45^{a}	27.67 ± 2.60^{b}	9.91 ± 1.45^{c}
	LMS	–	34.34 ± 3.56^{a}	12.50 ± 0.97^{b}
	HM	71.86 ± 5.28^{a}	60.32 ± 6.83^{b}	38.57 ± 1.99^{c}
δ-Tocopherol	LM	1.42 ± 0.10^{a}	1.05 ± 0.11^{b}	0.15 ± 0.06^{c}
	LMS	–	1.53 ± 0.18^{a}	0.49 ± 0.09^{b}
	HM	1.34 ± 0.05^{a}	1.18 ± 0.11^{b}	0.56 ± 0.05^{c}
α-Tocotrienol	LM	4.16 ± 0.49^{a}	3.02 ± 0.51^{b}	0.41 ± 0.14^{c}
	LMS	–	3.87 ± 1.39^{a}	0.91 ± 0.13^{b}
	HM	12.06 ± 1.24^{a}	10.18 ± 1.60^{b}	4.50 ± 0.34^{c}
γ-Tocotrienol	LM	6.09 ± 0.21^{a}	4.65 ± 0.31^{b}	1.25 ± 0.21^{c}
	LMS	–	5.65 ± 0.61^{a}	1.85 ± 0.15^{b}
	HM	15.45 ± 0.71^{a}	12.87 ± 0.93^{b}	7.03 ± 0.38^{c}
δ-Tocotrienol	LM	0.16 ± 0.02^{a}	0.16 ± 0.02^{a}	0.04 ± 0.02^{b}
	LMS	–	0.24 ± 0.04^{a}	0.09 ± 0.01^{b}
	HM	0.58 ± 0.05^{a}	0.54 ± 0.06^{a}	0.21 ± 0.04^{b}
Total tocochromanols	LM	58.58 ± 5.62^{a}	43.01 ± 4.57^{b}	13.23 ± 2.13^{c}
	LMS	–	52.82 ± 8.15^{a}	17.29 ± 1.37^{b}
	HM	150.93 ± 10.71^{a}	129.02 ± 14.74^{b}	71.77 ± 4.26^{c}
Lutein	LM	11.40 ± 1.08^{a}	9.95 ± 0.48^{b}	7.66 ± 0.85^{c}
	LMS	–	8.93 ± 0.33^{a}	7.39 ± 0.55^{b}
	HM	7.85 ± 0.19^{a}	7.21 ± 0.19^{a}	5.44 ± 0.72^{b}
Zeaxanthin	LM	9.23 ± 0.62^{a}	7.71 ± 0.41^{b}	6.60 ± 0.76^{c}
	LMS	–	6.92 ± 0.26^{a}	6.20 ± 0.37^{b}
	HM	4.73 ± 0.59^{a}	4.02 ± 0.17^{b}	2.95 ± 0.33^{c}
β-Cryptoxanthin	LM	0.82 ± 0.11^{a}	0.69 ± 0.03^{b}	0.60 ± 0.04^{b}
	LMS	–	0.63 ± 0.02 ^{a}	0.57 ± 0.02 ^{b}
	HM	0.75 ± 0.09^{a}	0.52 ± 0.02^{b}	0.45 ± 0.03^{b}
α-Carotene	LM	0.29 ± 0.04^{a}	0.18 ± 0.01^{b}	0.20 ± 0.02^{b}
	LMS	–	0.18 ± 0.01 ^{a}	0.19 ± 0.01 ^{a}
	HM	0.29 ± 0.02^{a}	0.26 ± 0.01^{b}	0.21 ± 0.01^{c}
β-Carotene	LM	0.71 ± 0.10^{a}	0.62 ± 0.01^{a}	0.66 ± 0.03^{a}
	LMS	–	0.61 ± 0.02^{a}	0.64 ± 0.02^{a}
	HM	0.99 ± 0.07^{a}	0.95 ± 0.02^{a}	

Table 1 (*continued*)

Compound	Sample material	Concentration [µg/g DW]		
		Flour	Porridge	Digesta
				0.79 ± 0.04^{b}
Total carotenoids	LM	22.45 ± 1.47^{a}	19.15 ± 0.92^{b}	15.71 ± 1.69^{c}
	LMS	–	17.27 ± 0.56^{a}	14.98 ± 0.92^{b}
	HM	14.61 ± 0.38^{a}	12.95 ± 0.37^{b}	9.84 ± 1.11^{c}

DW, dry weight. Concentration represented as mean ± standard deviation (n = 6). Values within rows not sharing a lower-case letter are significantly different ($p < 0.05$).

significant degradation of tocopherols nor of tocotrienols in the maize porridge during in vitro digestion.

3.2.2. α-Tocopherylquinone

α-Tocopherylquinone, an oxidation product of α-tocopherol, was identified in the maize flours, porridges and digesta by HPLC-MS. The sodium adduct of the molecular ion $[M + Na]^+$ at m/z 469 and the protonated molecular ion after dehydration $[M + H\text{-}H_2O]^+$ at m/z 429 were the predominant ions (Supplementary data, Fig. S1). The protonated molecular ion $[M + H]^+$ at m/z 447 was present at very low abundance. The described ions were in agreement with the finding of Tang et al. (2020). In addition, α-tocopherylquinone was distinguished from α-tocopherol by its lower UV absorption maximum at 268 nm.

In the flours of low phytic acid maize and high phytic acid maize, α-tocopherylquinone was found at concentrations of 4.47 µg/g of DW and 12.32 µg/g of DW, respectively (Fig. 2). Against expectation, the decrease in α-tocopherol after the production of maize porridge was accompanied by a decrease in α-tocopherylquinone to 0.37 µg/g of DW in the low phytic acid maize, 0.36 µg/g in the low phytic acid maize spiked with phytate and 4.69 µg/g of DW in the high phytic acid maize. A bell-shaped relationship was described for the formation of α-tocopherylquinone in sunflower oil at a temperature of 180 °C for up to 40 h (Rennick & Warner, 2006). In the described study, the maximum concentrations of α-tocopherolquinone were observed after a heating time of 20 to 30 h and then decreased during the subsequent 10 h of heating. Together with our findings, this suggests that α-tocopherylquinone may undergo consecutive reactions to other endproducts. After in vitro digestion, a significant increase in the α-tocopherylquinone concentrations was observed in all maize porridges, while α-tocopherol concentrations declined. Since α-tocopherylquinone in the digesta of low phytic acid maize with 1.79 µg/g of DW was in a similar concentration range as in the digesta of low phytic acid maize with admixed phytate with 2.02 µg/g of DW, there was no clear evidence that phytic acid may have prevented the oxidation of α-tocopherol. Although γ- or δ-tocopherol were significantly degraded from flour to digesta, their corresponding tocopherylquinones were not identified in the samples.

3.2.3. Carotenoids

The total carotenoid concentration in the high and low phytic acid maize samples decreased from flour to porridge and to the final digesta (Table 1) and dropped from 22.45 µg/g of DW and 14.61 µg/g of DW in the low phytic acid and high phytic acid maize flour to 19.15 µg/g of DW and 12.95 µg/g of DW in the respective porridges. The concentration in the porridge of low phytic acid maize spiked with phytate was slightly lower with 17.28 µg/g of DW compared to the low phytic acid maize porridge. The degradation order for low phytic acid maize with α-carotene > zeaxanthin > β-cryptoxanthin > lutein and β-carotene was comparable with the degradation order of low phytic acid maize spiked with phytate resulting in α-carotene > zeaxanthin > β-cryptoxanthin > lutein > β-carotene. However, undesired thermal isomerization of lutein and zeaxanthin, could have taken place as described by Kean et al.

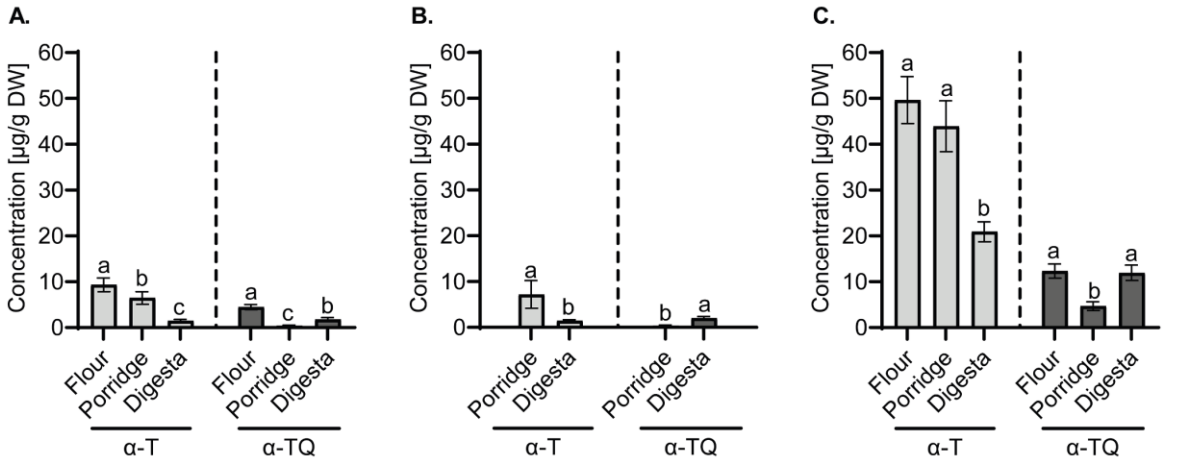

Fig. 2. α-Tocopherol (α-T) and α-tocopherylquinone (α-TQ) concentrations in flour, porridge, and digested maize porridge of low phytic acid maize (A), low phytic acid maize supplemented with phytate (B), and high phytic acid maize (C). *DW*, dry weight. Bars not sharing a lower-case letter are significantly different ($p < 0.05$).

(2008), the detection of which was outside of the capabilities of our method. In our study with a heating time of five minutes at a temperature of 90 °C, the largest degradation was observed for α-carotene with a loss of 37.9% in the low phytic acid maize as well as in the low phytic acid maize spiked with phytate after cooking. Besides the heating time, the degradation of carotenoids in orange maize flour was strongly affected by the moisture content revealing a possible loss in total carotenoids larger than 50% after heat and moisture treatments for more than 180 min (Beta & Hwang, 2018). In vitro digestion further decreased the concentrations of total and individual carotenoids in the maize porridges. Especially the reduction in provitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthin) resulted in similar concentrations in the digested low phytic acid maize porridge as in the digested low phytic acid maize porridge spiked with phytate. As observed for the tocochromanols, the addition of phytate did not show any remarkable effect on the degradation of carotenoids compared to the low phytic acid maize after cooking or in vitro digestion.

3.2.4. Fatty acids

Overall, the identified saturated fatty acids (palmitic, stearic, arachidic acid) and unsaturated fatty acids (oleic, linoleic, linolenic acid), determined as fatty acid methyl esters, in maize flour matched with those described previously in maize hybrids (Table 2) (Lux et al., 2021). The share in unsaturated fatty acids in the low phytic acid maize flour with 83.9% resembled the share in the high phytic acid maize flour with 84.3%. After porridge preparation, the share in total unsaturated fatty acids remained almost constant. A slight decrease in linoleic acid from 49.2% in the flour of low phytic acid maize to 47.8% in the porridge was observed accompanied by a marginal increase in oleic acid from 34.1% to 35.6%. The fatty acid composition of the low phytic acid maize flour was comparable with the composition in the low phytic acid flour with admixed phytate.

After in vitro digestion of the porridges, a significant decrease in the share of total unsaturated fatty acids was found resulting in 81.6% in the low phytic acid maize digesta, 80.8% in the low phytic acid maize supplemented with phytate and 82.2% in the high phytic acid maize digesta. This observation was caused by the significant decrease in oleic acid and linolenic acid. Except for high phytic acid maize, the share in linoleic acid was also decreased from porridge to digesta in the low phytic acid maize with and without admixed phytate. Especially during the intestinal phase, triglycerides were hydrolyzed by lipase resulting in an accumulation of fatty acids. In the case of unsaturated fatty acids, the free fatty acids were more prone to oxidation than their respective esters (Nieva-Echevarría et al., 2020). According to our hypothesis, a change in the unsaturated fatty acid profile, in particular of low phytic acid maize, after in vitro digestion was expected. However, the difference in the fatty acid profile between low and high phytic acid maize after in vitro digestion appear to be negligible and a preventive effect by the addition of phytate on unsaturated fatty acids was not achieved. Notably, the ether extract fraction was higher in the flour of the high phytic acid maize ("ÖL 4") with 122.9 g/kg compared to low phytic acid maize ("ÖL 3") with 69.9 g per kg (Rodehutscord et al., 2016), which may has evoked the difference in the degradation behavior of linoleic acid between low phytic acid maize spiked with phytate and the high phytic acid maize after in vitro digestion.

3.2.5. Malondialdehyde

Malondialdehyde equivalent concentrations were measured in the maize flours, porridges, and digesta to examine the effect of lipid oxidation after cooking and in vitro digestion. The concentrations of malondialdehyde equivalents in the low phytic acid maize flour were not different from the high phytic acid maize flour (Fig. 3). After cooking, the malondialdehyde equivalent concentrations remained constant. After the subsequent in vitro digestion of the maize porridges, a significant increase in the malondialdehyde equivalent concentration was observed for all samples resulting in 0.55 µg/g of DW in the low phytic acid maize digesta, 0.71 µg/g of DW in the digesta of low phytic acid maize admixed with phytate, and 0.63 µg/g of DW in the high phytic acid maize digesta. Even though the thiobarbituric acid reactive substance assay used in this study is widely applied to determine lipid oxidation in biological samples, the reader should be aware that this method has a limited specificity for malondialdehyde (Ghani et al., 2017). Increases in the malondialdehyde and 4-hydroxy-2-nonenal concentrations have also been reported in herring during the first 30 min of gastric digestion, reaching an equilibrium-like state between 30 and 90 min, followed by a lower formation of malondialdehyde during intestinal digestion (Larsson et al., 2016). According to the results in the maize flour with added phytate, a significant formation of malondialdehyde was accompanied by a decrease in the share in linoleic and linolenic acid after in vitro digestion. This observation was in conflict with the findings by Lee & Hendricks reporting an inhibitory effect by phytic acid on lipid peroxidation in a lipid model system with linoleic acid but the inhibition by phytic acid was dose-dependent with maximum phytic acid concentrations of 10 mmol/L (Lee & Hendricks, 1997). Hence, the natural difference in phytic acid between the genotypes may be too small to observe a measurable affect by phytic acid on

Table 2
Fatty acid composition in flour, porridge, and digested maize porridge of low phytic acid maize (LM), low phytic acid maize supplemented with phytate (LMS), and high phytic acid maize (HM).

Fatty acid	Sample material	Fatty acid composition [%]		
		Flour	Porridge	Digesta
Palmitic acid (16:0)	LM	12.9 ± 0.4^{b}	12.8 ± 0.1^{b}	14.1 ± 0.2^{a}
	LMS	–	12.5 ± 0.1^{b}	14.6 ± 0^{a}
	HM	12.2 ± 0.3^{b}	12.1 ± 0.6^{b}	13.7 ± 0.3^{a}
Stearic acid (18:0)	LM	2.1 ± 0^{b}	2.1 ± 0.1^{b}	3.1 ± 0.1^{a}
	LMS	–	2.1 ± 0.1^{b}	3.2 ± 0^{a}
	HM	2.2 ± 0.1^{b}	2.1 ± 0^{c}	2.8 ± 0.1^{a}
Arachidic acid (20:0)	LM	1.1 ± 0^{b}	1.1 ± 0^{b}	1.2 ± 0.1^{a}
	LMS	–	1.1 ± 0^{b}	1.3 ± 0^{a}
	HM	1.2 ± 0^{b}	1.1 ± 0^{c}	1.3 ± 0.1^{a}
Oleic acid (18:1*n*-9)	LM	34.1 ± 1.3^{ab}	35.6 ± 0.7^{a}	34.0 ± 0.9^{b}
	LMS	–	36.0 ± 0.2^{a}	35.2 ± 0.3^{b}
	HM	37.1 ± 1.5^{a}	36.7 ± 2.4^{a}	33.4 ± 1.0^{b}
Linoleic acid (18:2*n*-6)	LM	49.2 ± 1.1^{a}	47.8 ± 0.7^{b}	47.1 ± 0.7^{b}
	LMS	–	47.8 ± 0.4^{a}	45.1 ± 0.2^{b}
	HM	46.9 ± 1.2^{a}	47.5 ± 1.8^{a}	48.5 ± 0.7^{a}
Linolenic acid (18:3*n*-3)	LM	0.56 ± 0^{a}	0.56 ± 0^{a}	0.51 ± 0^{b}
	LMS	–	0.54 ± 0^{a}	0.47 ± 0^{b}
	HM	0.36 ± 0^{b}	0.40 ± 0^{a}	0.37 ± 0^{b}
Total saturated fatty acids	LM	16.1 ± 0.4^{b}	16.1 ± 0.2^{b}	18.4 ± 0.3^{a}
	LMS	–	15.7 ± 0.1^{b}	19.2 ± 0^{a}
	HM	15.7 ± 0.3^{b}	15.3 ± 0.6^{b}	17.8 ± 0.4^{a}
Total unsaturated fatty acids	LM	83.9 ± 0.4^{a}	83.9 ± 0.2^{a}	81.6 ± 0.3^{b}
	LMS	–	84.3 ± 0.1^{a}	80.8 ± 0^{b}
	HM	84.3 ± 0.3^{a}	84.7 ± 0.6^{a}	82.2 ± 0.4^{b}

Composition represented as mean ± standard deviation ($n = 6$). The fatty acid composition was given as percentage of fatty acid methyl esters in the sample. A standard deviation of zero represents a value smaller than 0.1. Values within rows not sharing a lower-case letter are significantly different ($p < 0.05$).

fatty acid oxidation.

3.3. *Digestive stabilities, solubilities, and micellarisation efficiencies of tocochromanols and carotenoids*

3.3.1. *Tocochromanols*

The digestive stability of total tocochromanols in the low phytic acid maize sample with 31.1% was similar with the digestive stability of the low phytic acid maize sample with admixed phytate with 33.4% but lower than the digestive stability of high phytic acid maize with a digestive stability of total tocochromanols of 56.5% (Table 3). Even higher stabilities were found in pasta with digestive stabilities of tocochromanols larger than 78% (Werner & Böhm, 2011). Except for δ-tocopherol, similar digestive stabilities were observed for individual tocopherols and tocotrienols in low phytic acid maize with admixed phytate compared to the stabilities in the low phytic acid maize sample without added phytate. The digestive stability of δ-tocopherol was on average 1.9-times higher in the low phytic acid maize with admixed phytate compared to the stability found in the low phytic acid maize sample but still 0.6-times lower than the digestive stability of δ-tocopherol in the high phytic acid maize. Since the digestive stability of tocochromanols between the low phytic acid maize spiked with phytate and high phytic acid maize were significantly different, compounds other than phytic acid might have influenced the stability of tocochromanols because the concentration of phytic acid in the flours of both samples were initially the same.

The significant differences between the high phytic acid maize and low phytic acid maize spiked with phytate were proceeded by the solubilities and micellarisation efficiencies of total and individual tocochromanols. A micellarisation efficiency of total tocochromanols of 10.5% was found in the high phytic acid maize sample. The micellarisation efficiencies of total tocochromanols in the low phytic acid maize samples with and without added phytate were comparable with 3.3% and 2.6%, respectively, and the difference was statistically not significant. Thus, the addition of phytate did not impact the bioaccessibility of tocochromanols in maize porridges in a considerable manner. In general, the micellarisation efficiencies of tocopherols were higher or equal to the micellarisation efficiencies of the respective tocotrienols, for instance the micellarisation efficiency of α-tocopherol with 9.8% was larger than the one of α-tocotrienol with 7.7% in the high phytic acid maize sample. Even higher micellarisation efficiencies were reached for tocochromanols in egg pasta with 49.4% (Werner & Böhm, 2011). According to the described study, the pH during the gastric phase and the amount of bile affected the bioaccessibility of tocochromanols which

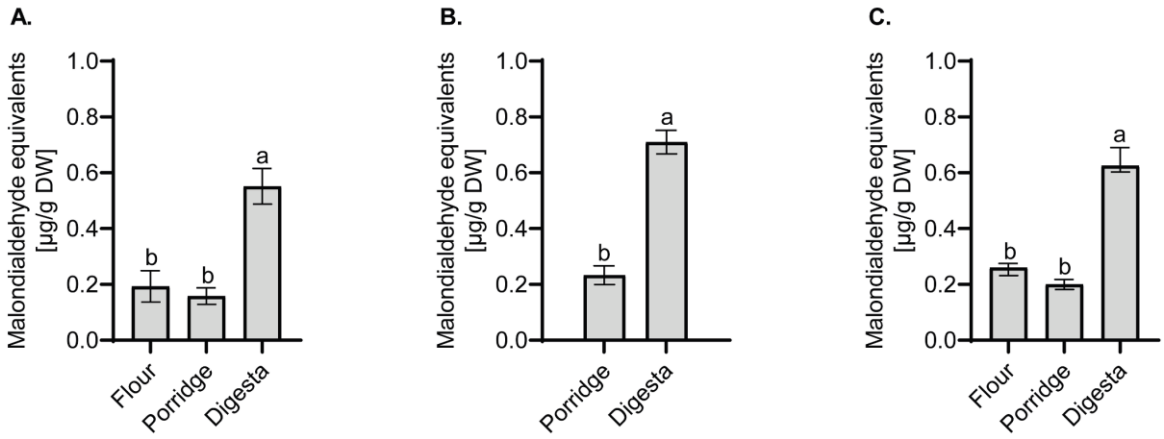

Fig. 3. Malondialdehyde equivalent concentrations in flour, porridge, and digested maize porridge of low phytic acid maize (A), low phytic acid maize with admixed phytate (B), and high phytic acid maize (C). *DW*, dry weight. Bars not sharing a lower-case letter are significantly different ($p < 0.05$).

Table 3
Digestive stabilities, solubilities, and micellarisation efficiencies of tocochromanols and carotenoids extracted from low phytic acid maize porridge (LM), low phytic acid maize porridge supplemented with phytate (LMS), and high phytic acid maize porridge (HM).

	Compound	LM	LMS	HM
Digestive stability [%]	α-Tocopherol	23.4 ± 6.0^{b}	23.5 ± 10.6^{b}	48.5 ± 10.1^{a}
	γ-Tocopherol	36.2 ± 7.1^{b}	36.7 ± 4.7^{b}	64.9 ± 10.0^{a}
	δ-Tocopherol	14.2 ± 6.1^{c}	32.6 ± 7.2^{b}	48.1 ± 8.3^{a}
	α-Tocotrienol	14.0 ± 5.2^{b}	26.3 ± 10.8^{b}	45.4 ± 9.5^{a}
	γ-Tocotrienol	27.1 ± 5.4^{b}	33.1 ± 4.3^{b}	54.9 ± 6.0^{a}
	δ-Tocotrienol	25.6 ± 12.9^{a}	37.5 ± 8.0^{a}	39.3 ± 10.7^{a}
	Total tocochromanols	31.1 ± 6.5^{b}	33.4 ± 5.2^{b}	56.5 ± 9.0^{a}
	Lutein	77.0 ± 8.1^{a}	82.9 ± 7.8^{a}	75.4 ± 8.9^{a}
	Zeaxanthin	85.6 ± 9.5^{ab}	89.7 ± 7.1^{a}	73.4 ± 7.3^{b}
	β-Cryptoxanthin	86.5 ± 4.7^{a}	90.4 ± 2.3^{a}	86.9 ± 6.1^{b}
	α-Carotene	109.2 ± 10.9^{a}	106.9 ± 8.8^{a}	82.0 ± 4.3^{b}
	β-Carotene	105.9 ± 5.7^{a}	104.4 ± 4.0^{a}	83.0 ± 4.8^{b}
	Total carotenoids	82.1 ± 8.3^{a}	87.8 ± 6.6^{a}	75.9 ± 7.7^{a}
Solubility [%]	α-Tocopherol	9.1 ± 4.2^{b}	7.4 ± 1.6^{b}	17.2 ± 4.0^{a}
	γ-Tocopherol	8.9 ± 4.3^{b}	3.8 ± 1.1^{b}	21.9 ± 4.3^{a}
	δ-Tocopherol	9.0 ± 4.6^{c}	2.1 ± 0.9^{b}	22.3 ± 3.4^{a}
	α-Tocotrienol	7.2 ± 3.8^{b}	5.1 ± 1.0^{b}	13.7 ± 2.3^{a}
	γ-Tocotrienol	6.5 ± 3.3^{b}	2.7 ± 0.9^{c}	14.8 ± 2.3^{a}
	δ-Tocotrienol	13.1 ± 8.6^{b}	2.9 ± 2.0^{c}	22.0 ± 3.6^{a}
	Total tocochromanols	8.6 ± 4.2^{b}	4.2 ± 1.2^{b}	19.0 ± 3.8^{a}
	Lutein	21.4 ± 2.9^{ab}	16.0 ± 2.1^{b}	23.1 ± 5.6^{a}
	Zeaxanthin	20.2 ± 2.8^{a}	15.3 ± 1.7^{a}	27.5 ± 5.9^{b}
	β-Cryptoxanthin	40.2 ± 1.8^{ab}	35.8 ± 2.0^{b}	42.3 ± 5.0^{a}
	α-Carotene	70.6 ± 6.2^{a}	71.5 ± 1.9^{a}	68.2 ± 4.5^{a}
	β-Carotene	58.4 ± 2.8^{a}	56.4 ± 2.4^{ab}	51.8 ± 5.4^{b}
	Total carotenoids	23.8 ± 3.0^{ab}	18.9 ± 2.0^{b}	28.5 ± 5.8^{a}
Micellarisation efficiency [%]	α-Tocopherol	3.7 ± 0.7^{b}	5.0 ± 0.7^{b}	9.8 ± 1.7^{a}
	γ-Tocopherol	3.5 ± 0.7^{b}	2.2 ± 0.6^{b}	12.0 ± 2.4^{a}
	δ-Tocopherol	3.3 ± 0.8^{b}	1.0 ± 0.5^{b}	13.3 ± 2.7^{a}
	α-Tocotrienol	2.3 ± 0.7^{b}	3.6 ± 0.5^{b}	7.7 ± 1.3^{a}
	γ-Tocotrienol	2.4 ± 0.5^{b}	1.5 ± 0.5^{b}	8.3 ± 1.6^{a}
	δ-Tocotrienol	3.7 ± 1.5^{b}	1.0 ± 1.1^{b}	12.6 ± 3.1^{a}
	Total tocochromanols	3.3 ± 0.7^{b}	2.6 ± 0.5^{b}	10.5 ± 2.0^{a}
	Lutein	18.5 ± 2.1^{a}	14.1 ± 1.2^{b}	12.4 ± 2.3^{b}
	Zeaxanthin	17.5 ± 2.2^{a}	13.7 ± 0.9^{b}	16.6 ± 2.6^{ab}
	β-Cryptoxanthin	37.4 ± 3.2^{a}	32.3 ± 1.9^{b}	35.1 ± 1.9^{ab}
	α-Carotene	–	–	63.9 ± 2.5
	β-Carotene	56.6 ± 3.8^{a}	53.6 ± 3.0^{a}	45.1 ± 1.5^{b}
	Total carotenoids	20.2 ± 2.3^{a}	16.2 ± 1.1^{b}	18.4 ± 2.5^{ab}

DW, dry weight. Digestive stability, solubility, and micellarisation efficiency are represented as mean ($n = 6$) ± standard deviation. α-Carotene was not detected in the micellar phases of low phytic acid maize with or without added phytate. Values within rows not sharing a lower-case letter are significantly different ($p < 0.05$).

may be one reason for the discrepancy. According to Table S1b in the supplementary material of Rodehutscord et al. (2016), the high phytic acid maize "ÖL 4" contained more lipophilic compounds, determined as ether extract, than the low phytic acid maize "ÖL 3". Thus, the elevated concentration in lipophilic compounds might have facilitated the formation of micelles, resulting in a higher solubilisation of lipophilic compounds (Porter et al., 2007).

3.3.2. Carotenoids

In the case of carotenoids, the average digestive stabilities of total carotenoids ranged from 56.5% in the high phytic acid maize porridge to 31.1% in the low phytic acid maize porridge (Table 3). From this perspective, it appears that the higher phytic acid concentration had evoked a protective effect, but the digestive stabilities of total carotenoids of the low phytic acid maize porridge with added phytate were also low with 33.4%, disproving the hypothesis.

Contrarily, the micellarisation efficiencies of total carotenoids were significantly higher in the low phytic acid maize porridge with 20.2% compared to the digested maize porridge with added phytate with 16.2%. The micellarisation efficiency of total carotenoids in the high phytic acid maize porridge with 18.4% was in between. Thus, it appeared that phytic acid lowered the micellarisation efficiency of most carotenoids in maize porridge. In an experiment with protein-stabilized oil-in-water emulsions, aggregation of oil droplets occurred by the addition of phytic acid during emulsion preparation, showing that the physical stability was impacted by phytic acid altering the charge characteristics of the droplets under acidic conditions (Pei et al., 2020). In our in vitro digestion experiments, the pH was shifted from pH 2.5 in the gastric phase to pH 6.5 in the intestinal phase. The different pH conditions in combination with phytic acid addition could have reduced the micellarisation efficiency of carotenoids. Even though the addition of phytic acid significantly lowered the micellarisation efficiency, it should be emphasized that the effect was relatively small.

In the three maize porridge samples, the mean micellarisation efficiencies of β-carotene (45.1% to 56.6%) were greater than the mean micellarisation efficiencies of the xanthophylls lutein (12.4% to 18.5%) and zeaxanthin (13.7% to 17.5%). An enhanced micellarisation of carotenes, in particular of α-carotene in maize-based porridges compared to maize bread was found in a previous article (Kean et al., 2008). In this regard, the authors interpreted that the food matrix affected the inclusion of carotenoids into micelles during digestion. Hence, food matrix effects should be considered, when comparing the micellarisation efficiencies of low and high phytic acid maize.

4. Conclusion

In conclusion, tocochromanols, carotenoids, and unsaturated fatty acids in maize were decreased during cooking and subsequent simulated digestion. Especially after in vitro digestion, the concentrations of total tocochromanols and total carotenoids decreased, while simultaneously

the concentrations of the oxidation products α-tocopherylquinone and malondialdehyde equivalents increased. This suggests the occurrence of prooxidative processes during in vitro digestion.

The addition of phytic acid to maize flour did not impact the digestive stability of tocochromanols and carotenoids in maize porridge, but phytic acid addition significantly decreased the micellarisation efficiencies of total and individual carotenoids in maize porridge, perhaps due to a reduced physical stability of the micelles. However, further research (in vitro digestion experiments and human trials) is warranted to investigate if an excess of phytate might prevent oxidation reactions in maize during digestion. For this purpose, different concentrations of phytate could be added to the test meals to investigate dose–response relationships.

CRediT authorship contribution statement

Peter E. Lux: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Larissa Fuchs:** Investigation, Software, Data curation, Writing – review & editing. **Nina Wiedmaier-Czerny:** Methodology, Writing – review & editing. **Jan Frank:** Supervision, Project administration, Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132053.

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Supplementary material

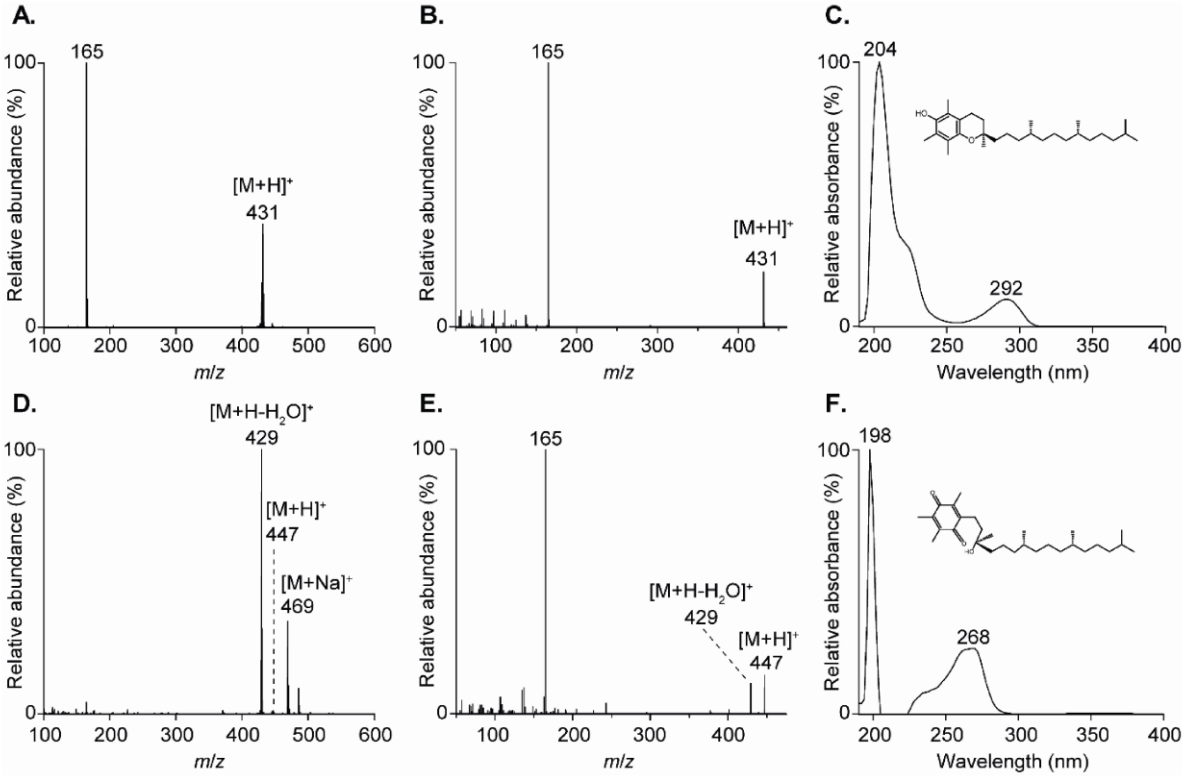

Figure S1 Full scan mass spectra, MS^2 spectra, and UV spectra of an α-tocopherol standard (A, B, C) and α-tocopherylquinone standard (D, E, F) using the applied APCI(+)-MS parameters. $[M+H]^+$, protonated molecular ion. $[M+Na]^+$, protonated sodium adduct of the molecular ion. $[M+H-H_2O]^+$, protonated molecular ion after dehydration.

Table S1 Zinc and iron concentrations in flour, porridge, and digested maize porridge of low phytic acid maize, low phytic acid maize supplemented with phytate, and high phytic acid maize

Mineral	Sample material	Concentration [µg/g DW]		
		Flour	Porridge	Digesta
Zinc	LM	19.42 ± 0.46	20.02 ± 0.71	20.63 ± 0.38
	LMS	-	19.90 ± 1.02	20.78 ± 0.33
	HM	19.78 ± 0.34	19.18 ± 0.21	20.93 ± 0.69
Iron	LM	20.65 ± 0.71	21.62 ± 0.55	22.53 ± 0.54
	LMS	-	21.55 ± 1.33	23.72 ± 1.45
	HM	25.22 ± 0.81	26.35 ± 4.84	25.30 ± 1.07

DW, dry weight. *HM,* high phytic acid maize. *LM*, low phytic acid maize. *LMS*, low phytic acid maize supplemented with phytate. Concentration represented as mean ± standard deviation (n = 6).

Table S2 Concentration of tocochromanols in the liquid digesta, aqueous phase, and micellar phase of digested low phytic acid maize porridge, low phytic acid maize porridge supplemented with phytate, and high phytic acid maize porridge

Tocochromanol	Sample material	Concentration [ng/mL]		
		Digesta	Aqueous phase	Micellar phase
α-Tocopherol	LM	286.6 ± 1.2	26.2 ± 12.2	10.7 ± 1.9
	LMS	318.1 ± 0.7	23.4 ± 4.9	15.9 ± 2.3
	HM	1818.1 ± 5.4	312.1 ± 73.2	176.8 ± 30.1
γ-Tocopherol	LM	1226.5 ± 5.2	109.3 ± 53.1	43.0 ± 8.4
	LMS	1517.9 ± 3.3	57.1 ± 17.3	33.5 ± 9.3
	HM	2495.8 ± 7.4	547.2 ± 107.9	299.4 ± 61.1
δ-Tocopherol	LM	46.8 ± 0.2	4.2 ± 2.2	1.6 ± 0.4
	LMS	67.6 ± 0.1	1.4 ± 0.6	0.7 ± 0.3
	HM	48.6 ± 0.1	10.8 ± 1.6	6.5 ± 1.3
α-Tocotrienol	LM	133.8 ± 0.6	9.7 ± 5.1	3.0 ± 0.9
	LMS	171.0 ± 0.4	8.7 ± 1.7	6.2 ± 0.9
	HM	421.0 ± 1.2	57.9 ± 9.9	32.4 ± 5.7
γ-Tocotrienol	LM	206.1 ± 0.9	13.5 ± 6.9	5.0 ± 1.0
	LMS	249.5 ± 0.5	6.7 ± 2.2	3.8 ± 1.1
	HM	532.7 ± 1.6	78.7 ± 12.2	44.3 ± 8.8
δ-Tocotrienol	LM	7.0 ± 0	0.9 ± 0.6	0.3 ± 0.1
	LMS	10.4 ± 0	0.3 ± 0.2	0.1 ± 0.1
	HM	22.2 ± 0.1	4.9 ± 0.8	2.8 ± 0.7
Total tocochromanols	LM	1906.8 ± 8.2	163.7 ± 80.0	63.4 ± 12.7
	LMS	2334.4 ± 5.1	97.5 ± 26.9	60.2 ± 12.8
	HM	5338.4 ± 15.9	1011.5 ± 203.1	562.1 ± 106.6

DW, dry weight. *HM,* high phytic acid maize. *LM*, low phytic acid maize. *LMS*, low phytic acid maize supplemented with phytate. Concentration represented as mean ± standard deviation (n = 6 digestions).

Table S3 Concentration of carotenoids in the liquid digesta, aqueous phase, and micellar phase of digested low phytic acid maize porridge, low phytic acid maize porridge supplemented with phytate, and high phytic acid maize porridge

Carotenoid	Sample material	Concentration [ng/mL]		
		Digesta	Aqueous phase	Micellar phase
Lutein	LM	407.0 ± 45.1	86.0 ± 5.4	74.4 ± 6.1
	LMS	392.6 ± 29.4	62.3 ± 5.2	55.1 ± 1.6
	HM	301.9 ± 40.0	68.1 ± 10.4	37.0 ± 5.7
Zeaxanthin	LM	350.7 ± 40.4	69.9 ± 3.9	60.8 ± 4.2
	LMS	329.1 ± 19.4	50.1 ± 3.8	45.0 ± 1.3
	HM	163.7 ± 18.6	44.3 ± 6.0	26.9 ± 3.1
β-Cryptoxanthin	LM	31.7 ± 2.0	12.7 ± 0.6	11.8 ± 0.5
	LMS	30.5 ± 1.2	10.9 ± 0.7	9.8 ± 0.6
	HM	24.9 ± 1.4	10.5 ± 1.0	8.7 ± 0.2
α-Carotene	LM	10.4 ± 1.0	7.3 ± 0.1	-
	LMS	10.0 ± 0.3	7.1 ± 0.1	-
	HM	11.7 ± 0.5	8.0 ± 0.3	7.5 ± 0.2
β-Carotene	LM	35.0 ± 1.8	20.4 ± 0.3	19.8 ± 0.7
	LMS	33.7 ± 1.5	19.0 ± 0.6	18.1 ± 0.5
	HM	43.7 ± 2.1	22.5 ± 1.6	19.7 ± 0.7
Total carotenoids	LM	834.7 ± 89.5	196.3 ± 9.9	166.8 ± 11.2
	LMS	795.8 ± 48.8	149.5 ± 9.9	128.0 ± 3.0
	HM	545.8 ± 61.4	153.4 ± 19.2	99.7 ± 9.6

DW, dry weight. *HM,* high phytic acid maize. *LM*, low phytic acid maize. *LMS*, low phytic acid maize supplemented with phytate. Concentration represented as mean ± standard deviation (n = 6 digestions).

Chapter 5

General discussion

With a growing world population and limited global P-resources, it is of utmost importance to close the P-cycle in agriculture and to ensure food security on our planet (Cordell et al., 2009; Nedelciu et al., 2020). In addition, it is necessary to use P more efficiently and targeted in agricultural cropping systems to overcome P-limitation on crops and in particular in maize, which has a high P-demand compared to other cereals (Müller & Zhang, 2019). Consequently, outcomes of P-limitation on compounds with nutritional relevance in maize need to be better understood. As part of the AMAIZE-P project, it was hypothesized in this dissertation that nutrients, in particular fatty acids, phenolics, carotenoids, and tocochromanols in maize (*Zea mays* L.) grains are affected by a reduced availability of phosphate in soil during cultivation. Furthermore, changes in the chemical composition of the maize grains, especially in phytic acid, may affect the oxidative stability of maize-based products during processing and digestion.

The effects of phosphate fertilization and sowing time on (poly)phenol, carotenoid, and tocochromanol concentrations in maize (*Zea mays* L.) grains

To work towards the **first aim**, which was the investigation of the influence of a reduced phosphate-availability on (poly)phenols, carotenoids, and tocochromanols in maize grains, an experimental site for the cultivation of maize was preselected with plant-available P-concentration < 3 mg CAL-P/100 g soil, representing a low P-status in soil (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2018). A site nearby Freising with 1.6 mg CAL-P/100 g soil met the criterion and was consequently selected for this field experiment. One commercially available maize genotype was grown in a randomized field experiment without (P0) or with the application of 44 kg P/ha (P44) as triple superphosphate (TSP) to achieve a difference in P-availability. As additional factor, the grains were sown at two different dates spaced by 21 days and then harvested on the same day. Prior to the analyses of the grains, the extraction and HPLC method for carotenoids was adapted to allow the measurement of elevated concentrations of lutein and zeaxanthin. In addition, different parameters of the extraction of carotenoids from food sources were optimized for ground maize grains, including saponification times (30 min), saponification

temperatures (38 °C), and extraction solvents (*n*-hexane/diethyl ether (50/50, *v*/*v*)). The chosen conditions increased the extraction of carotenoids from maize towards recovery rates > 90%. Finally, P, (poly)phenol, carotenoid, and tocochromanol concentrations in the grains were quantitatively analyzed (Chapter 2; Lux et al., 2020). The resulting key findings of chapter 2 were as follows:

- A remarkable variation of (poly)phenols (10 insoluble and 19 soluble (poly)phenols) were identified in these maize grains, including phenolic amines and di- and trimers of ferulic acid.
- Total and individual insoluble and soluble (poly)phenol concentrations were not significantly ($p < 0.05$) different comparing grains of P0 with P44 groups, but they were individually affected by the sowing time.
- Lutein, zeaxanthin, β-cryptoxanthin, α-, and β-carotene were detected in the grains, but their concentrations were not significantly influenced by the application of TSP within the group of early or late sown maize plants.
- Significantly higher concentrations of lutein, zeaxanthin, β-cryptoxanthin and β-carotene were observed in grains of late sown compared to the early sown maize plants.
- Among tocochromanols, γ- and δ-tocopherols and tocotrienols were detected in the maize grains, whereof γ-tocopherol was the most abundant tocochromanol
- Total tocochromanol concentrations in the grains of maize plants were unaffected by fertilization with TSP during cultivation.
- Mean P-concentrations in the maize grains of plants grown with TSP tended to be slightly higher (3.20 mg/g of DW in the early and 3.11 mg/g of DW in the late sown maize grain samples) than the control (3.02 mg P/g of DW in the early and 2.91 mg P/g of DW in the late sown maize grain samples).

The identified and quantified soluble and insoluble (poly)phenols, carotenoids, and tocochromanols in chapter 2 were in agreement with described literature data. Interestingly, α-tocopherol was not detected in the maize grain material. On the contrary, α-tocopherol has been found in maize grains of 4786 maize lines with median concentrations of 8.68 µg/g, when grown under standard agronomic practices (Diepenbrock et al., 2017). The absence of α-tocopherol in the maize grains could be explained by the following principle: In the first phase of plant stress response in leaves, α-tocopherol is increasingly synthesized followed by a second phase

characterized by tocopherol degradation (Munné-Bosch, 2005). Thus, the analysis of α-tocopherol oxidation products, in particular α-tocopherylquinone, may confirm that α-tocopherol was synthesized in the maize grains but was subsequently oxidized, and, therefore, not detectable. Other authors reported an increase in the formation of malondialdehyde, another oxidation product, and damaging hydrogen peroxide in maize leaves of phosphate-deficient maize seedlings (Zhang et al., 2014), supporting this idea.

The insignificant impact of P-availability on (poly)phenol, carotenoid, and tocochromanol concentrations in maize grains raised the questions whether the plants were supplied with sufficient P during growth. The mean concentrations of P in the grains were above 2.8 mg P/g of DW, which was selected as an upper limit for P-deficiency according to the study of Roberts & Rhee (1993). Maize plants have evolved a number of strategies to enhance P-availability. For instance, in maize grown under low P-conditions, proteins for phosphate transporters and acid phosphatases were increasingly expressed in the roots (Nie et al., 2021). Under severe nutrient deficiency, maize plants can also increase the efflux of malate and citrate from the roots as a mechanism to enhance P-acquisition and recruit beneficial rhizobacteria (Jones & Darrah, 1995; Wu et al., 2018). As a P-starvation response, plant cells can also remodel their membranes replacing phospholipids with sulfolipids or galactolipids to recycle P (Cruz-Ramirez et al., 2006; Essigmann et al., 1998). These P-uptake strategies and the redistribution of P within the plant by membrane remodeling cannot be excluded in this study and may be the reason for the balanced P-concentrations of the grains of the unfertilized control and fertilized maize plants.

In a two-year fertilization experiment on a site with a similar P-concentration in the soil (2.99 mg CAL-P/100 g soil), but at a P-application of 80 kg P/ha, applied with seven different P-containing fertilizers, including phosphate rock, no significant differences in the shoot P-uptake for maize were reported between the unfertilized control and the treatment samples (Wollmann & Möller, 2018). This observation gave a first indication, that even at higher P-application rates compared to the rate used in Chapter 2, only marginal differences regarding the P-uptake are expected under these conditions. Nevertheless, the tendency that the P-concentrations in the grains can be slightly enhanced by P-fertilization in maize plants, is consistent with a published study, in which the P and phytate concentrations in the grains were increased with a rising

P-application rate (10, 26, and 70 mg P/kg applied as superphosphate) during cultivation (Modi & Asanzi, 2008).

Besides, one could hypothesize that the CAL method did not catch all fraction of plant-available P in the tested soil, because details on the P-fractions that are extracted by the CAL method are still lacking, and, therefore, may give a lower plant-available P-concentration. This hypothesis has partly been confirmed, because phytates that contribute to the organic fraction of soil, are insufficiently represented by the CAL method (Steffens et al., 2010). However, in another study it was experimentally demonstrated that the CAL method compared to the Olsen method overestimates the concentration of plant-available P, mostly by extracting $Ca_3(PO_4)_2$ more efficiently from soils with a high sorption capacity (Hartmann et al., 2019). The different extraction efficiencies for P-fractions by the different P-extraction methods (for instance, Olsen < CAL < Mehlich 3) was also confirmed by another study (Wuenscher et al., 2016). This suggests that at least the same extraction method for the analysis of plant-available P in soil (here CAL-P) should be considered, when comparing the results of chapter 2 with other publications.

The accumulative effect of a later sowing time was most distinctive for total carotenoid concentrations (late sown P0 grains ≈ late sown P44 grains > early sown P0 grains ≈ early sown P44 grains), which was mostly attributed to differences by the xanthophylls lutein and zeaxanthin (Lux et al., 2020). Elevated total carotenoids were recently reported for 24 late sown genotypes from three species, with an average accumulation of carotenoids for barley with 12.2%, bread wheat with 15.7%, and durum wheat with 27.8%, which was explained by higher thermal and water stress for the crops (Beleggia et al., 2021). These observations together with the results described in chapter 2 indicate that the effect of the sowing time on carotenoids may be an interspecies effect.

Overall, this is the first study under in vivo conditions that revealed the larger impact by the timing of sowing on (poly)phenols, carotenoids, and tocochromanols in the grains of this maize variety compared to phosphate fertilizer application. The presence of (poly)phenols, carotenoids, and tocochromanols in the maize grains of the maize hybrid cultivated under low P-conditions gives first evidence that they were still synthesized by the plant under the described conditions, emphasizing their importance in this reproductive body.

The interactions between phosphate fertilization, place of cultivation, and variety on fatty acids, carotenoids, and tocochromanols in maize (*Zea mays* L.) grains

Since the first study was limited to one maize variety, a second study was started to test the hypothesis with a larger gene pool and to address the **second aim**, which included the identification of possible interactions between phosphate fertilization, the location, and the maize variety and their single effects on the analytes (fatty acids, carotenoids, and tocochromanols) in the grains. Eight commercially available maize hybrids within early to medium-early maturity groups were chosen to reduce differences in the concentration of lipophilic compounds evoked by different maturity stages at harvest. These varieties were planted in replicated plots without phosphate fertilization or with phosphate fertilization (52.9 kg P/ha), applied as TSP, at three sites in Southern Germany (Stuttgart-Hohenheim, Eckartsweier, and Dettingen) with well-supplied soils. (Poly)phenols were excluded in the second study due to their lower relevance for humans, as described in Chapter 2.2.2, while fatty acids were included as analytes. Fatty acids, carotenoids, and tocochromanols were quantified in the grains and statistically analyzed using a mixed model (Chapter 3; Lux et al., 2021). Intraday and interday repeatability were assessed to ensure a high precision of the analytical methods. The key results of Chapter 3 were described and discussed below.

- Saturated (palmitic, stearic, arachidic) and unsaturated (oleic, linoleic, linolenic) fatty acids as fatty acid methyl esters (FAME), tocochromanols (α-, β-, γ-, δ-tocopherols and -tocotrienols), and carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene) were detected in the grains of eight maize hybrids.
- The effects of fertilization with 52.9 kg P/ha applied as TSP and interactions between phosphate fertilization and the place of cultivation (location) on the fatty acid composition, carotenoid and tocochromanol concentrations in grains of maize plants were considered as insignificant ($p < 0.05$).
- Maize grains of hybrids cultivated at the site with the highest mean temperature (Eckartsweier with 11.7 °C) had a significantly higher proportion of stearic acid and oleic acid and a lower proportion of linoleic acid and linolenic acid compared to the grains from hybrids cultivated at the site with the lowest mean temperature (Dettingen with 9.1 °C)

- Significant differences in total provitamin A carotenoid concentrations (β-cryptoxanthin, α- and β-carotene) in the grains were found between the eight maize genotypes with highest median concentrations in Ricardinio (7.2 µg/g of DW) and lowest median concentration in Amaveritas (2.1 µg/g of DW), while the highest median concentrations of total tocochromanols were found in the variety LG 30.258 (94.9 µg/g of DW) and lowest in P8329 (57.9 µg/g of DW).
- With the exception of γ-tocopherol, significant interactions between the variables maize grain variety and the place of cultivation on the concentration of individual tocochromanols and carotenoids and on the proportions of fatty acids were identified.

The main fatty acids, carotenoids, and tocochromanols in the eight maize hybrids were identified and were comparable to literature data described in Chapter 3. In comparison to the phosphate-fertilized plants, the fatty acid profile of the maize grains from control plants remained constant (Chapter 3, Table S3). Fertilization experiments conducted on a soil with a moderate P-availability (6.5 mg P/100 g soil) with a mixture of nitrogen, potassium, and P showed a slight change in the fatty acid composition of rape seeds (*Brassica napus* L.), with an increase in arachidic and eicosenoic acid, while the proportions of palmitic, stearic, oleic, linoleic, and linolenic acid remained unaffected (Załuszniewska & Nogalska, 2020). More pronounced effects were found in a long-term field experiment of maize with a combined application of nitrogen, potassium, and P as a fertilizer. In this study a significant shift in the fatty acid profile towards a higher content of unsaturated fatty acids and a decrease in the content of saturated fatty acids was highlighted. These results were attributed to potassium as main limiting factor on oil quality (Ray et al., 2019). This gives reason to suggest the control of plant nutrient concentrations other than P in soil, such as potassium, to avoid influences by these nutrients, when assessing the impact of P-availability on the fatty acid composition in maize.

Combining the results of Chapter 2 and 3, it can be concluded that P-fertilization applied as TSP did not significantly enhance the concentrations of carotenoids and tocochromanols in these maize grains when cultivated on soils with P-availabilities ranging from 1.6 to 20.6 mg CAL-P/100 g soil (soil classification B to E). Interestingly, interactions between phosphate application and the location or the variety did also not significantly impact the concentrations of total carotenoids and total tocochromanols in

the maize grains (Lux et al., 2021). Effects on these organic compounds in maize grown on soils with extremely low P-concentrations, corresponding to soil classification A (< 1.5 mg CAL-P/100 g soil) described by the Association of German Agricultural and Analytic Research Institutes (Verband Deutscher Landwirtschaflticher Untersuchungs- und Forschungsanstalten, 2018), could still be possible. Nevertheless, only 3% of the total arable land in Germany has a plant-available phosphate concentration < 2.0 mg CAL-P/100 g of soil (Römer & Steingrobe, 2018; Werner, 2014). This shows in turn that the present results are applicable to most of Germany´s cropland. Thus, in Germany, phosphate fertilizer input could potentially be reduced for the described maize genotypes in the short term when only (poly)phenols, fatty acids, carotenoids, and tocochromanols are the target factor. Nevertheless, P-concentrations in soil and within the crop should be checked on regular intervals in order to monitor the removal of P from the soil.

Differences between the locations, which combined multiple factors, such as climatic conditions and soil properties, evoked significant changes in the proportion of fatty acids (stearic, oleic, linoleic, and linolenic acid) as well as in the concentrations of provitamin A carotenoids (β-cryptoxanthin and β-carotene), and tocochromanols (γ- and δ- tocopherols and tocotrienols), when the statistical mixed model was applied (Lux et al., 2021). Even though these changes appear to be marginal, these results become more meaningful in the context of climate change, including extreme weather events, which recently has gained more attention in food production (Gomez-Zavaglia et al., 2020).

The significant differences in the fatty acid composition as well as carotenoid and tocochromanol concentrations between the maize varieties point out, that farmers can actively select the maize variety for seeding, depending on the specific requirements of the grains for subsequent food or feed processing. The influence of the variety ($p < 0.001$) on the concentration of these organic compounds in maize was more pronounced than the effect of the location or phosphate application (Lux et al., 2021). Nevertheless, side-effects of phosphorus compounds on the bioaccessibility of carotenoids and tocochromanols should be verified, which was partially examined in the next section.

The impact of phytic acid on the oxidative stability of tocochromanols, carotenoids, and fatty acids in maize (*Zea mays* L.) porridges during cooking and digestion

As described in the introduction section, the major proportion of P in maize grains is stored as phytic acid, which is considered as antinutrient and may in addition prevent oxidation (Empson et al., 1991; Rodehutscord et al., 2016; Urbano et al., 2000). Before proposing to breed for more P-efficient maize lines that produce either low or high phytic acid crops, it is important to achieve a better understanding of the function of phytic acid on the oxidative stability of maize-based foods. Thus, the **third aim** was addressed which comprised the investigation of phytic acid on the stability of (unsaturated) fatty acids, carotenoids, and tocochromanols, and the formation of oxidation products (malondialdehyde, α-tocopherylquinone) during cooking and simulated digestion of maize porridge. Thereby, a subgoal was the examination of the influence of phytic acid on digestive parameters (digestive stability, solubility, and micellarization efficiency) of carotenoids and tocochromanols in maize porridge.

Maize grain flours (ground maize grains) and porridges were obtained from high (HM; 16.6 µmol/g DW) or low (LM; 10.9 µmol/g of DW) phytic acid maize genotypes. Additionally, maize porridge with added phytic acid as sodium phytate was prepared from the low phytic acid maize flour (LMS), yielding the same phytic acid concentration as the high phytic acid maize. An in vitro digestion protocol including an oral phase with α-amylase addition was chosen to simulate the physiological digestion conditions of starchy products (Thakkar et al., 2007). Thus, three-stage in vitro digestion experiments were conducted with the maize porridges based on a protocol used for sorghum (Lipkie et al., 2013). Aqueous and micellar phases were obtained from the digesta. Fatty acids, tocochromanols, carotenoids, and oxidation products were analyzed in the flours, porridges, and digesta. To this end, a new extraction and HPLC-MS method for the analysis of α-tocopherylquione was developed and validated. Digestive stabilities, solubilities, and micellarization efficiencies of carotenoids and tocochromanols were assessed (Chapter 4, Lux et al., 2022). The key findings of Chapter 4 were described and discussed below.

- Cooking reduced the mean phytic acid concentrations by 7% and 13% for low and high phytic acid maize flour, respectively, and increased minor inositol

phosphates (mainly *myo*-inositol-1,2,4,5,6-penta*kis*phosphate) in maize porridge.

- A significant ($p < 0.05$) decrease in total tocochromanol (α-, γ-, δ-tocopherols and -tocotrienols) and total carotenoid (lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene) concentrations were observed in LM, LMS, and HM after cooking and in vitro digestion.
- The proportion of total unsaturated fatty acids (oleic, linoleic, linolenic acid), determined as FAME, was significantly reduced after in vitro digestion of porridge prepared from LM, LMS, and HM.
- During digestion of porridge prepared from LMS, a significant reduction in α-tocopherol from 7.2 to 1.5 µg/g of DW was accompanied by an increase in the oxidation products α-tocopherylquinone from 0.4 µg/g of DW to 2.0 µg/g of DW and malondialdehyde from 0.2 µg/g of DW to 0.7 µg/g of DW suggesting that phytic acid at the admixed dose did not effectively prevent oxidation.
- The addition of phytic acid did not significantly change the digestive stabilities of total tocochromanols and total carotenoids whereas the micellarization efficiencies of total carotenoids were significantly lower for LMS with 16.2 ± 1.1% compared to porridge prepared from LM with 20.2 ± 2.3%.

From the results of Chapter 4 it can be concluded that phytic acid did not prevent the degradation of tocochromanols, carotenoids, and fatty acids after cooking and subsequent in vitro digestion in maize in a significant manner. This was also reflected by the digestive stability of total and individual carotenoids and tocochromanols, for instance α-tocopherol with average digestive stabilities of 23.4% in LM porridge and 23.5% in porridge prepared from LMS. However, the observed differences in the digestive stabilities of individual tocochromanols and carotenoids of LMS compared to HM indicated that other compounds excluding phytic acid may be involved in this degradation process (Lux et al., 2022). Nevertheless, the simultaneous increase in oxidation products (malondialdehyde, α-tocopherylquinone) in porridges prepared from LM, LMS, and HM gave evidence that oxidation processes occurred in these maize samples during in vitro digestion. These oxidative conditions may be facilitated by the acidic conditions during the gastric phase as well as the presence of metal ions coming from the food (Nieva-Echevarría et al., 2020). Although α-, γ-, and δ-tocopherols degraded in the maize samples during cooking and in vitro digestion, only α-tocopherylquinone was detected among the tocopherylquinones using the here

developed HPLC-MS method (Lux et al., 2022). Thus, an extension in the number of tocopherol oxidation markers is recommended when assessing the oxidation status of tocopherols in a maize-based product during in vitro digestion. For instance, 3,7,11-trimethyl-3-dodecanol and 4,8,12,16-tetramethylheptadecan-4-olide, which were formed from tocopherols under accelerated storage conditions in corn oil, were suggested as potential oil oxidation markers and could be analyzed in future studies (Alberdi-Cedeño et al., 2019).

As a side effect of porridge preparation, a slight degradation of the phytic acid concentration concomitant with an increase in metabolites of phytic acid was found (Lux et al., 2022). This finding may be attributed to the intrinsic phytase activity of the utilized maize grain material, which was determined in a previous study (Rodehutscord et al., 2016). When cooking was combined with addition of 1.2% lime, a process termed nixtamalization, a phytic acid degradation between 4.4% and 27.9% was achieved for whole maize grains, while the reduction was even higher for the isolated maize endosperm fraction with degradation rates up to 59.8% (Bressani et al., 2002). Moreover, steam pressure treatments at a pressure of 2.5 MPa for 60 s resulted in an average phytic acid degradation of roughly 87% in wheat bran (Guo et al., 2015). This revealed the high potential of sample pre-processing when aiming at a reduction in phytic acid concentration in foods.

Another key conclusion is that phytic acid significantly reduced the micellarization efficiency of total and individual carotenoids, substantiating the antinutrient properties of phytate (Lux et al., 2022). A reduced micellarization of β-carotene from green leafy vegetables caused by the addition of ferulic acid and catechin was explained by a reduction in the lipase activity with 21.3% and 13.6%, respectively (Kruger et al., 2019). For phytic acid, a partial inhibitory effect at a phytic acid concentration of 4 mmol/L and at pH 6.5 on the pancreatic lipase activity was elucidated (Knuckles, 1988), which could be the reason for the reduced micellarization efficiency for most carotenoids in chapter 4. It was also reported that phytic acid (0.05% to 0.3%, *w*/*w*) can affect the ζ-potential under acidic conditions, which led to aggregation of oil droplets in protein-stabilized oil-in-water emulsions by charge neutralization and, thereby, decreased its physical stability (Pei et al., 2020). Since the electric charge of mixed micelles can affect their interaction with SR-B1 and CD36, which are involved in the transport of these micelles across the intestinal membrane (Goncalves et al., 2015), the uptake of

micellarized carotenoids obtained from digested LM, LMS, and HM should be examined in cell culture studies.

In general, the results of chapter 4 highlighted for the first time, that phytic acid added to maize flour during processing, did not significantly improve the digestive stability of total carotenoids and tocochromanols. This was accompanied by a decrease in the proportion of total unsaturated fatty acids during digestion. In the case of α-tocopherol, the degradation was supported by the increase in the concentrations of the rarely investigated α-tocopherylquinone in the maize porridges during digestion. Furthermore, phytic acid added to the maize sample decreased the micellarization efficiency of carotenoids (Lux et al., 2022). This may result in an impaired bioavailability of carotenoids in humans who incorporate high amounts of foods rich in phytic acid in their daily diet. Human trials should follow to test different concentration of phytic acid on the digestive stability and assess the bioavailability of carotenoids and tocochromanols of maize-based products in vivo.

Relevance, limitations, and future research opportunities

This dissertation gave a first insight into the influence of soil P-availability during growth of maize plants on (poly)phenols, fatty acids, carotenoids, and tocochromanols in their grains. When only considering these organic compounds, a reduction in phosphate fertilization for these maize hybrids could be implemented on the majority of German fields without significantly affecting the concentrations of these organic compounds (Chapter 2 and 3). Since the studies were limited to one-year field experiments, long-term field experiments with maize cultivated without phosphate fertilizer should be conducted to clarify if specific conditions (for instance an extremely low concentration of plant-available phosphate in the soil) will be arising over time which could restrict the biosynthesis of these organic compounds in the grains. In order to control the concentrations of fatty acids, carotenoids, and tocochromanols, the applied HPLC-(MS) or GC(-MS) analyses, which require labor-intensive sample preparation and long analyses time, could be replaced by faster methods and analyzed on the field at harvest. For instance, near-infrared spectroscopy (NIRS) seems to be a promising technique for the rapid prediction of fatty acids, carotenoids, and tocopherols in cereals, which has recently been tested for ground maize (Egesel et al., 2016; Kahrıman et al., 2019). Breeding of P-efficient maize genotypes that can also adopt to

other environmental stressors would be another approach to balance possible effects on grain quality and stabilize yields (Lux et al., 2021).

In addition, the intracellular P-dynamics in maize grown under P-deficiency need to be clarified in detail, before unraveling the function of P in the biosynthesis of these organic compounds in maize on a cellular basis. Therefore, the recent approach with sensors of the fluorescence indicator protein for inorganic phosphate family, which were expressed in *Arabidopsis thaliana* seedlings and used for live imaging of P-distribution by laser scanning or spinning disc confocal microscopy, could be transferred to maize (Assunção et al., 2020; Mukherjee et al., 2015).

Finally, the reduction of the micellarization efficiency of carotenoids in maize porridge by phytic acid is a novel observation that contributes to the scientific knowledge of the effects of phytic acid as antinutrient (Chapter 4). Whether minor phosphates (inositol penta-, tetra, and triphosphates) in processed maize also contribute to a reduced micellarization efficiency or affect the oxidative stability by binding non-complexed minerals to a significant degree during digestion should be evaluated in further simulated digestion studies. This assumption is supported by the fact that pH-dependent differences in mineral complexation (zinc, copper, and cadmium) between inositol pentaphosphate and lower inositol phosphates were observed (Persson et al., 1998). The performed digestion experiments could be complemented by measuring non-complexed and complexed metals by phytic acid and minor inositol phosphates at each stage of the digestion process. Finally, human bioavailability studies would be required to confirm the observed effects of phytic acid in vivo, before giving dietary recommendations regarding phytic acid concentrations for maize.

Overall, this dissertation interconnected the global challenge in agriculture to reduce phosphate fertilization in maize production with its nutritional consequences, such as maintaining high quality grains for human nutrition. Furthermore, the knowledge about the multiple functions and effects of phytic acid, using simulated digestion, was substantially extended and brings impulses for further research.

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Contributions to publications

M.Sc. Peter Erwin Lux adjusted and validated the method for the extraction and HPLC analyses of carotenoids in Chapter 2; acquired the maize samples from the Technical University of Munich used for the experiments in Chapter 2; performed the extraction and HPLC-(MS) analyses of (poly)phenols and tocochromanols in Chapter 2, and carotenoids in Chapter 2 and 4; participated on the harvest of the maize grain samples used in Chapter 3; performed the extraction and analyses of FAME in Chapter 3; was involved in the HPLC analyses of tocochromanol and carotenoids in Chapter 3 as well as in the analyses of α-tocopherylquinone, tocochromanols, malondialdehyde, FAME and determination of phytase activity presented in Chapter 4; conducted parts of the digestion experiments in Chapter 4; developed and validated the method for the extraction and analyses of α-tocopherylquinone by HPLC-MS in Chapter 4; performed most of the statistical analyses of the results in Chapter 2, 3, and 4; created the figures and wrote the first drafts of the manuscripts reprinted in Chapters 2, 3, and 4.

Prof. Dr. Jan Frank actively supervised the dissertation and contributed to the conceptualization and design of the experiments described in Chapters 2, 3, and 4; edited and co-authored the manuscripts of Chapters 2, 3, and 4; was responsible for the funding acquisition.

Dr. Wolfgang Stuetz was involved in defining the overall research aim and supported the carotenoid analyses by HPLC in Chapter 2; co-authored the manuscript of Chapter 2.

Dr. Sabine von Tucher and **M.Sc. Markus Freiling** planed and conducted the field experiment of maize in Chapter 2 and provided information about the soil status and P-concentrations in the grains; both co-authored the manuscript of Chapter 2.

Prof. Dr. Dr. h.c. Reinhold Carle provided the equipment for the (poly)phenol analyses and contributed to the editing of the manuscript in Chapter 2.

Dr. Christof Björn Steingaß actively supported the method development of the (poly)phenol analysis by HPLC-MS and their identification described in Chapter 2; edited and co-authored the manuscript of Chapter 2.

Prof. Dr. Walter Vetter provided the equipment for the GC analyses in Chapter 3 and 4 and contributed to the editing of the manuscript in Chapter 3 as co-author.

M.Sc. Nina Wiedmaier-Czerny and **M.Sc. Franziska Müller** adjusted the method for the analysis of FAME by GC and co-authored the manuscript of Chapter 3. **M.Sc. Nina Wiedmaier-Czerny** actively supported the identification of FAME by GC in Chapter 4 and reviewed the manuscript.

Prof. Dr. Tobias Würschum and **M.Sc. Thea Mi Weiß** planed and conducted the field experiments described in Chapter 3 together with their field team and contributed as co-authors to the manuscript of Chapter 3; both conducted parts of the statistical analysis in Chapter 3.

M.Sc. Alice-Jaqueline Reineke performed the soil analysis and characterization in Chapter 3.

M.Sc. Jeanine Schneider participated on the extraction and HPLC analysis of carotenoids and tocochromanols in Chapter 3; co-authored the manuscript in Chapter 3.

Dr. Jens Pfannstiel and his team gave technical assistance at the LC-MS analysis in Chapter 4.

M.Sc. Larissa Fuchs produced the maize porridges; conducted parts of the digestion experiments and extracted the tocochromanols, FAME, α-tocopherylquinone, and malondialdehyde described in Chapter 4; co-authored the manuscript in Chapter 4.

M.Sc. Moritz Novotny performed the analysis of inositol phosphates in Chapter 4.

12.05.2022

Further activities during the doctoral thesis

Supervision of students and their contributions

2020. Jeanine Schneider, M.Sc.

Master student in Molecular Nutrition Science, University of Hohenheim.

Title of Profile Area Experimental Project in Nutritional Science: High drying temperature reduces concentrations of carotenoids and vitamin E in yellow maize (*Zea mays* L.) grains during post-harvest drying

Title of the thesis: Tocopherol, tocotrienol, and carotenoid profiles in maize grains as influenced by phosphate fertilization, genotype, and location of cultivation

2021. Larissa Fuchs, M.Sc.

Master student in Nutritional Medicine, University of Hohenheim.

Title of Profile Area Experimental Project in Nutritional Science: Residence time of the oral phase does not have a crucial impact on the digestive stabilities of tocochromanols during in vitro digestion of maize (*Zea mays* L.) porridge

Title of the thesis: Influence of phytate on tocochromanols, fatty acids, and oxidation products thereof in maize (*Zea mays* L.) porridges after cooking and in vitro digestion

International cooperations

2019. Xiuxiu Chen, M.Sc.

Guest researcher from China Agricultural University

Topic: Concentrations of carotenoids and vitamin E in wheat cultivated under different phosphate conditions in China

2019. Xiaohong Sun, M.Sc.

Guest researcher from China Agricultural University

Topic: Carotenoids and tocochromanols in germ, endosperm, and pericarp of Chinese maize varieties

Participation in symposia

7th Annual Nutrition Omics Symposium – Advances, Applications, and Translations in Nutrition and Epidemiology. 2021. (virtual participation).

Recent Advances in Food Analysis. 2021. (virtual participation).

Contributions to Scientific Conferences

Peter E. Lux, Wolfgang Stuetz, Jan Frank. (2021). The impact of phosphate-availability and phytate on tocochromanols and carotenoids in maize. Sino-German Conference on Maize-Based Phosphate Cycles in Agriculture, University of Hohenheim, Germany.

AMAIZE-P

The impact of phosphate-availability and phytate on tocochromanols and carotenoids in maize

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Introduction

Phosphorus (P) is a macronutrient for plants. In maize, P is needed, for instance as geranyl geranyl diphosphate, for the biosyntheses of tocochromanols and carotenoids. Some of these plant metabolites are essential micronutrients for humans, such as α-tocopherol (vitamin E). In maize grains, P is also stored as phytate, which chelates minerals and may inhibit iron-catalysed oxidation. Thus, it was hypothesised, that the concentrations of tocochromanols and carotenoids in maize kernels are influenced by phosphate availability. We also aimed to unravel the indirect „antioxidant" effect of phytate in maize porridges on tocochromanols and carotenoids during in vitro digestion.

Material and Methods

For the first goal, grains of eight maize varieties grown with 52.9 kg of P per ha or without starter fertiliser at three sites in Germany (provided by RS 1.1) were chosen. Carotenoids and tocochromanols were determined in extracts of ground grains. Secondly, maize porridges were prepared from ground low-phytate maize grains (10.9 µmol/g of DW, provided by RS 3.2) with or without addition of sodium phytate. Porridges were digested using a human in vitro digestion model. Carotenoids, tocochromanols, and α-tocopherylquinone, an oxidation product of α-tocopherol, were analysed in the porridges and digesta fractions. Digestive stabilities and micellarisation efficiencies of carotenoids and tocochromanols were calculated.

Results

Total tocochromanols and carotenoids in maize grains were not significantly (p < 0.05) affected by phosphate ferilisation when grown on soils with oridinary P concentrations. After digestion of maize porridges prepared from low-phytate maize with or without the addition of sodium phytate, carotenoids and tocochromanols, including α-tocopherol, degraded in both groups while α-tocopherylquinone concentrations increased. Thus, phytate addition did not prevent a significant degradation of carotenoids and tocochromanols but decreased the micellarisation efficiency of carotenoids.

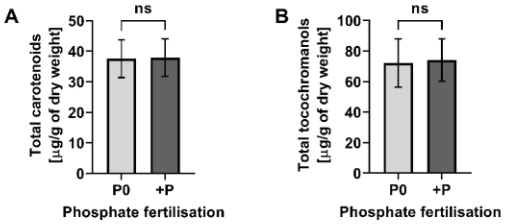

Figure 1: Mean concentrations of total carotenoids (A) and total tocochromanols (B) in grains of eight maize hybrids cultivated each in replicated plots with 52.9 kg P per ha (+P) or without (P0) phosphate starter fertiliser at Hohenheim, Eckartsweier, and Dettingen. *ns*, not significant. *t*-test was performed at p < 0.05.

Table 1: Concentrations, digestive stabilities, and micellarisation efficiencies of carotenoids and tocochromanols in maize porridges prepared from low-phytate maize with or without addition of phytate

	Concentration [µg/g of dry weight]			
	Low phyt maize		Low phyt maize + phyt	
Compound	Porridge	Digesta	Porridge	Digesta
Total carotenoids	19.2 ± 0.9 a	15.7 ± 1.7 b	17.3 ± 0.6 a	15.0 ± 0.9 b
Total tocochromanols	43.0 ± 4.6 a	13.2 ± 2.1 b	52.8 ± 8.2 a	17.3 ± 1.4 b
α-Tocopherol	6.5 ± 1.4 a	1.5 ± 0.3 b	7.2 ± 3.0 a	1.5 ± 0.2 b
α-Tocopheryl-quinone	0.4 ± 0.2 b	1.8 ± 0.4 a	0.4 ± 0.2 b	2.0 ± 0.3 a

	Digestive stability [%]		Micellarisation efficiency [%]	
Compound	Low phyt maize	Low phyt maize + phyt	Low phyt maize	Low phyt maize + phyt
Total carotenoids	82.1 ± 8.3 a	87.8 ± 6.6 a	20.2 ± 2.3 a	16.2 ± 1.1 b
Total tocochromanols	31.1 ± 6.5 a	33.4 ± 5.2 a	3.3 ± 0.7 a	2.6 ± 0.5 a

Phyt, phytate. Concentration shown as mean (n = 6) ± standard deviation. *t*-test was conducted. Different letters (*a*, *b*) indicate signifcant differences (p < 0.05) between means. Total carotenoids are the sum of lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene. Total tocochromanols comprise α-, γ-, δ- tocopherols and -tocotrienols.

Conclusion / Outlook

Long-term field experiments with very low phosphate concentrations should follow to confirm that the effect of phosphate availability on carotenoid and tocochromanol concentrations in maize grains is negligible from a nutritional point-of-view. Nevertheless, nutritional parameters such as the micellarisation efficiency of carotenoids were decreased by phytate.

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Curriculum vitae

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List of publications

1) **Lux, P. E.**; Fuchs, L.; Wiedmaier-Czerny, N. & Frank, J. (2022). Oxidative stability of tocochromanols, carotenoids, and fatty acids in maize (*Zea mays* L.) porridges with varying phytate concentrations during cooking and in vitro digestion. *Food Chemistry,* 378, 132053.

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Stuttgart, 11.05.2022

Place, date

Peter Lux

Signature

Declaration of authorship

Anlage 3

Eidesstattliche Versicherung über die eigenständig erbrachte Leistung

gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema

The influence of phosphate-availability and phytic acid on the profiles of fatty acids, (poly)phenols, carotenoids, and tocochromanols in maize (*Zea mays* L.) grains – from field experiments to human in vitro digestion studies

handelt es sich um meine eigenständig erbrachte Leistung.

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