TECHNISCHE UNIVERISTÄT MÜNCHEN

Fakultät für Chemie

Impact of Malting of Barley and Wheat on the Formation and Release of the Precursors of Desired and Undesired Vinyl Aromatics during the Production of Wheat Beer

Valerian Kalb

Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Disseration.

Vorsitzender:

Prof. Dr. Michael Rychlik

Prüfende der Dissertation:

1. Prof. Dr. Michael Granvogl
2. Prof. Dr. Wilfried Schwab

Die Dissertation wurde am 29.11.2021 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 15.03.2022 angenommen.

Acknowledgement

I would like to seize this opportunity to thank all the people who made this thesis possible.

My deep gratitude goes to Prof. Dr. Michael Granvogl, my PhD supervisor for entrusting this interesting, challenging, and very varied research topic to me. I want to thank him for the great guidance throughout the whole work, the confidence he put into me and coupled to that, the great freedom I had to try and learn what ever I was interested in, the countless working hours on correcting manuscripts and the strong backing when facing obstacles. I really appreciated the great teamwork and that time was never limited when it came to discussions. I further want to thank him for the opportunities to present my work to the professional public at various conferences, with the highlight going to the ACS National Meeting in San Diego. Apart from his expertise in food chemistry, I had the pleasure to benefit from his enormous treasure on travel recommendations.

This project was financed by the Association for the Promotion of Science of the German Brewing Industry (Wifoe), which should be honored at this point. A great thanks goes also to the members of the project-related committee for their support and to the project partners from the Chair of Brewing Science and Beverage Technology at the Technische Universität Berlin for their collaboration. Especially Torsten Seewald has to be mentioned, who was co-founder of the research topic, responsible for the preparation of malt and wort samples, helped when it came to brewing related questions and welcomed me for 2 days in his lab, where I could put hands on beer brewing on a 1 hL plant.

I'd like to thank all technical assistants who contributed to this work, notably Anneliese Köhler, who spent many hours on sample preparation, Ines Otte for the operation of the GCxGC-ToF-MS, and lust but not least Sami Kaviani-Nejad, who was in charge of the HPLC-MS/MS measurements.

In addition, I want to thank Dr. Johanna Kreißl for the introduction into the various GC systems, Richard Hammerl for the help with the interpretation of the NMR experiments, and Jörg Stein for his immense knowledge in chemical synthesis and what ever else was needed. Great thanks further goes to Prof. Corinna Dawid, Angela Lebedicker, Dr. Oliver Frank, Dr. Timo Stark and Dr. Stefan Klade, who supported me wherever they could.

Dr. Maximilian Dietz, Dr. Klaas Reglitz, and Benedikt Bächler shared with me the passion for the science of beer brewing and were the contact point to discuss results, exchange knowledge, and sometimes just to drink beer.

Many thanks go to the members of the ACS student chapter at TUM. It was a great time organizing interesting talks, start-up seminars, statistic courses, and awesome excursions to Austria, Switzerland, and elsewhere. Being a member of this group opened the doors for a broader understanding of food chemistry not only in the context of science.

What would be life at university without people to connect, to celebrate success, who motivate you when things aren't working, who share with you the passion for science, and who are a family for you far away from home. This family was found in the entire group of the Chair of Food Chemistry and Molecular Sensory Science and the group of the Leibniz Institute for Food Systems Biology at the Technical University of Munich. A special thanks goes to my lab mates from lab U.23/5 Susanne Wudy, Dr. Xiaoting Zhai, Florian Utz, and Florian Ziegler and my room mates Carolina Schury and Dr. Sabrina Brendel, a day in the lab with all of you was never boring. Further, lunches would never have been that funny without the Joint-Lunch-Circle: Silvia Lang, Caterina Porcelli, Stefanie Fischer, Angelika Gabler, Sarah Jöstl, and Robin Maier. Finally, a big thank to the "Gang" Dr. Alin Kraus, Dr. Karin Seebald, Daniela Füllemann, Dr. Annika Schulz, and Dr. Gerold Rebholz for all their support, talks, ideas, and motivation.

At this point, I would like to thank Jasmine Kalb for her moral assistance and endurance to listen to my presentations for training. Her immense passion for her own topic motivated me day after day. A big thank also goes to my parents Birgit and Edwin Kalb for supporting me morally and financially through all these years. Thanks also to my sisters Katherina and Leonie Kalb for bringing this craziness into my live. At the end, I also want to thank my friend Lorenz Bachmeier for emptying my stock of wine during hours of discussions, he really trained me in how to prepare arguments when going into a discussion or when presenting a topic.

Thank you!

Preliminary Remarks

The experimental part of the present work was performed between July 2017 and September 2020 at the Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Freising, Germany, under the supervision of Prof. Dr. Michael Granvogl (Technical University of Munich (July 2017 – April 2019) and University of Hohenheim (April 2018 – present)). Required brewing experiments were performed at the Chair of Brewing Science and Beverage Technology at the Technische Universität Berlin, Berlin, Germany, under the direction of Torsten Seewald.

Publications

Major parts of this thesis were published in international, peer-reviewed journals and presented at national and international symposia.

Kalb, V.; Hofmann, T.; Granvogl, M. Abhängigkeit der Freisetzung von Zimtsäure vom Grad des Zellwandabbaus während der Mälzung von Gerste und Weizen. September 17-19, **2018**, Poster presentation, 47. Deutscher Lebensmittelchemikertag, Berlin, Germany.

Kalb, V.; Hofmann, T.; Granvogl, M. Bildung erwünschter Schlüsselaromastoffe in Weizenbier - ein Vergleich zum toxikologisch relevanten Styrol. March 11-12, **2019**, Oral presentation, Regionalverband Bayern 70. Arbeitstagung, Erlangen, Germany.

Kalb, V.; Hofmann, T.; Granvogl, M. Styrene, the undesired and toxicologically relevant brother of the desired key aroma compounds of wheat beer. Abstracts of Papers, 258th ACS National Meeting & Exposition, San Diego, CA, United States, August 25-29, **2019**, AGFD-282.

Kalb, V.; Granvogl, M. Formation of desired smoky key odorants in wheat beer: a comparison with the undesired toxicologically relevant styrene. In *Sex, Smoke, and Spirits: The Role of Chemistry*; ACS Symposium Series 1321; Guthrie, B., Beauchamp, J. D., Buettner, A., Toth, S., Qian, M. C., Eds.; American Chemical Society: Washington, DC, United States, **2019**; pp 93–105.

Kalb. V.; Hofmann, T.; Granvogl, M. Impact of malting on the formation and release of desired and undesired precursors of vinyl aromatics during the production of wheat beer. May 11, **2020**, Oral presentation, Forschungsseminar der Lebensmittelchemie, Freising, Germany.

Kalb, V.; Hofmann, T.; Granvogl, M. Formation of desired key odorants in wheat beer - a comparison with the undesired toxicologically relevant styrene. February 11. **2020**, Oral presentation, 53. Brautechnologisches Seminar, Freising, Germany.

Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Studies on the impact of malting and mashing on the free, soluble ester-bound, and unsoluble ester-bound forms of desired and undesired phenolic acids aiming at styrene mitigation during wheat beer brewing. *J. Agric. Food Chem.* **2020**, *68*, 12421–12432.

Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The role of endogenous enzymes during malting of barley and wheat varieties in the mitigation of styrene in wheat beer. *J. Agric. Food Chem.* **2020**, *68*, 13888–13896.

Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The malting parameters: steeping, germination, withering, and kilning temperature and aeration rate as possibilities for styrene mitigation in wheat beer. *Eur. Food Res. Technol.* **2021**, https://doi.org/10.1007/s00217-021-03852-5.

Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Investigations into the ability to reduce cinnamic acid as undesired precursor of toxicologically relevant styrene in wort by different barley to wheat ratios (grain bill) during mashing. *J. Agric. Food Chem.* **2021**, *69*, 9443–9450.

Abstract

Wheat beer is a unique beer type, predominantly brewed in the German speaking areas of Europe, Germany and Austria. With a production volume of approximately 9 million hL in Germany and 0.2 million hL in Austria in 2018, wheat beer made up 9.2 and 2.0%, respectively, of the annual aggregated beer output in these countries. Traditionally, this beer type is brewed from malts of barley and wheat (>50%) using top fermenting yeasts. Thereby, the aroma-active vinyl aromatics 4-vinylphenol and 2-methoxy-4-vinylphenol are formed, giving wheat beer its very popular and characteristic, clove-like and slightly phenolic aroma expected by the consumers.

The aforementioned desired key aroma compounds in wheat beer are formed in a decarboxylation process from cinnamic acid derivatives (traditionally referred to as phenolic acids), namely *p*-coumaric and ferulic acid, in presence of Pof-active top-fermenting yeasts. To this biochemical process cinnamic acid, also originating from the used malts of barely and wheat, is a precursor, leading to the formation of the toxicologically relevant, and thus, undesired styrene. Known since the 1970s to be a natural component in wheat beer, its presence was ignored until 1996, when the World Health Organization (WHO) published a tolerable daily intake (TDI) for drinking water of 7.7 μg/kg body weight per day. At the latest with the classification as "possibly carcinogenic to humans" (class 2B) by the International Agency for Research on Cancer (IARC), the brewing industry became aware of this topic and put efforts on styrene mitigation to protect consumers safety.

The present thesis joined a series of studies on styrene mitigation and aimed at closing the knowledge gap on the influence of malting of barley and wheat on the formation and release of the precursors of desired and undesired vinyl aromatics during the production of wheat beer. The first part of this thesis focused on a basic understanding of the fate of these phenolic acids during malting and mashing. For that purpose, malt and wort samples were prepared from four barley and three wheat varieties using standard conditions typically applied during wheat beer production. Grain, malt, and wort samples of these experiments were then screened for their total, soluble, and free cinnamic, *p*-coumaric, and ferulic acid contents. The results showed that cinnamic acid determines less than 0.1% of the total phenolic acid content in grain and malt and less than 2% in wort. The at the same time comparable concentrations of free cinnamic and *p*-coumaric acid in grain and malt could be attributed to the fact that cinnamic acid was

already present in malt to more than 80% in a soluble form while *p*-coumaric and ferulic acid were still to more than 90% in an insoluble ester-bound form. The high percentage of free cinnamic acid compared to *p*-coumaric and ferulic acid also had a direct effect on the transfer rates from malt to wort, which were greater than 100% for cinnamic acid and less than 8% for the desired precursors. A comparison of barley and wheat malts with regard to their transfer of cinnamic, *p*-coumaric and ferulic acids into the wort showed that the use of barley malts leads to a transfer in a ratio of 2:14:84 and of wheat malts to 1:2:97. Next, with the objective to get a deeper insight into the enzyme activities, directly or indirectly involved in the release of phenolic acids during malting, grain and malts of the same sample set were screened for their α- and β-amylase, β-glucanase, feruloyl esterase, and protease activities. Additionally, total and water-extractable arabinoxylan contents as well as β-glucan contents were analysed, due to the fact that the target phenolic acids are preliminary bound to arabinoxylan. Based on a complete degradation of the β-glucan and thus of the cell walls, the inclusion of phenolic acids in intact cell walls and thus their inaccessibility by enzymes could be excluded. As expected, there was a positive correlation between the degradation of arabinoxylan and the release of phenolic acids. Interestingly, however, there was no direct relationship between the activity of feruloyl esterase, which is responsible for the direct release of phenolic acids, and the free phenolic acid concentration, indicating that the feruloyl esterase activity is not exclusively responsible for the free phenolic acid content and that further factors such as inhibitor proteins and the total phenolic acid contents must be taken into account. The absence of correlations between the protease activity and the enzyme activities investigated also showed that the enzymes involved in the release of phenolic acids are not targeted by the proteases.

Based on the new findings in the second part of this thesis, different selected malting parameters, such as the steeping temperature, the germination temperature in combination with the aeration rate, and the temperatures applied during kiln-drying, were evaluated for their suitability for styrene mitigation. For each of these parameters, multiple malting experiments were performed using the same raw material consisting of two barley varieties and one wheat variety. From the malts, wort were prepared to study how changes upon malting affected the precursor concentrations in the corresponding wort. The greatest potential for styrene reduction was shown by the withering temperature. When malting barley and wheat, lower temperatures should be preferred over higher temperatures. On a laboratory scale, a reduction of the styrene concentration of up to 72% could be achieved by selecting lower withering temperatures. The second largest influence was shown by the germination temperature together with the aeration rate, whereby in this case higher temperatures and lower aeration rates had a favourable effect on a reduction of styrene with a reduction potential of up to 52%. The smallest influence was shown by the steeping temperature, with the ability to reduce the styrene concentration by up to 16% by using higher temperatures. Interestingly, the malting parameter with the highest process temperature also showed the greatest influence, which was assumed to be due to the temperature dependence of the enzymes.

So far, barley and wheat varieties were always studied separately; thus, in the third part of this thesis, wort was prepared from different barley to wheat malt ratios. Data from these experiments were used to determine if and how phenolic acid contents and enzymatic activities in these grain types interact with each other, when mixing them for wheat beer brewing. Thereby, it was shown that there are no synergistic effects when barley and wheat malts are blended during mashing. Instead, a linear correlation was found depending on the ratio between barley and wheat malt and the phenolic acid concentrations. The phenolic acid content in wort used for wheat beer production is thus a function of the concentration in wort made from 100% barley or wheat multiplied by the respective percentage of the grain type used for mashing. Finally, on the basis of the yeast's own feruloyl esterase activity and the missing correlation between the free phenolic acid contents in process intermediates to the vinyl aromatic contents in wheat beer, wort was further fermented in presence of two different Pof-active top-fermenting yeast strains, to answer the question to what extent soluble ester-bound phenolic acids in wort contribute to the final vinyl aromatic contents in wheat beer. With comparable concentrations of soluble ester-bound phenolic acids in wort an beer, it was shown that during fermentation no additional release of soluble ester-bound phenolic acids and consequently no conversion to the corresponding vinyl aromatics took place. At the same time, a complete degradation of the free phenolic acids has been observed, demonstrating that the final vinyl aroma concentrations are mainly determined by the free phenolic acid concentrations in the pitching wort rather than by the contents of soluble ester-bound phenolic acids or the yeast's own feruloyl esterase.

Kurzfassung

Weizenbier ist ein spezieller Biertyp, welcher vor allem im deutschsprachigen Raum Europas (Deutschland und Österreich) gebraut wird. Mit einem Produktionsvolumen von 9 Millionen hL in Deutschland und 0,2 Millionen hL in Österreich im Jahr 2018 betrug der Anteil von Weizenbier am Jahresbierausstoß in diesen Ländern 9,2 bzw. 2,0 %. Traditionell wird dieser Biertyp aus Gersten- und Weizenmalz (>50 %) unter Einsatz von obergärigen Hefen hergestellt, wobei aromaaktive Vinylaromaten gebildet werden, welche dem Weizenbier das beliebte und charakteristische Nelken-artige und leicht phenolische Aroma verleihen.

Die oben genannten erwünschten Schlüsselaromastoffe in Weizenbier werden in einem Decarboxylierungsprozess aus Zimtsäurederivaten (traditionell als Phenolsäuren bezeichnet), nämlich *p*-Cumar- und Ferulasäure, in Gegenwart von Pof-aktiven, obergärigen Hefen gebildet. In diesem biochemischen Prozess wird auch Zimtsäure, welche ebenfalls aus den eingesetzten Gersten- und Weizenmalzen stammt, umgesetzt, wobei unerwünschtes, toxikologisch relevantes Styrol entsteht. Seit den 1970er Jahren ist bekannt, dass Styrol ein natürlicher Bestandteil von Weizenbier ist. Jedoch erlangte sein Vorkommen erst 1996 Beachtung, als die Weltgesundheitsorganisation (WHO) eine tolerierbare tägliche Aufnahme (TDI) von 7,7 μg/kg Körpergewicht und Tag festlegte. Spätestens mit der Klassifizierung als „möglicherweise krebserregend beim Menschen“ (Klasse 2B) durch die Internationale Agentur für Krebsforschung (IARC) wurde die Brauindustrie auf dieses Thema aufmerksam und setzte große Anstrengungen in die Styrolminimierung aus Gründen des Verbraucherschutzes.

Die gegenwärtige Dissertation basiert auf einer Reihe von Studien zur Styrolminimierung und war im Speziellen auf die Aufklärung der Einflüsse der Vermälzung von Gerste und Weizen auf die Ausbildung und Freisetzung der Präkursoren von erwünschten und unerwünschten Vinylaromaten bei der Herstellung von Weizenbier fokussiert.
Der erste Teil dieser Arbeit zielte auf ein grundlegendes Verständnis der Veränderungen, welche infolge des Mälzens und Maischens an den Phenolsäuren auftreten, ab. Dazu wurden Malz- und Würzeproben unter typischen Bedingungen der Weizenbierproduktion aus vier Gersten- und drei Weizensorten hergestellt und die unvermälzten Korn-, Malz- und Würzeproben auf deren Gehalt an totaler, löslicher und freier Zimt-, *p*-Cumar und Ferulasäure untersucht. Die Ergebnisse zeigten, dass Zimtsäure in Korn und Malz weniger als 0,1 % und in der Würze weniger als 2 % des Gesamtphenolsäuregehaltes bestimmt. Die gleichzeitig vergleichbaren Konzentrationen

von freier Zimt- und *p*-Cumarsäure in Korn und Malz konnten dabei darauf zurückgeführt werden, dass Zimtsäure bereits in Malz zu über 80 % in einer löslichen und *p*-Cumar- und Ferulasäure noch zu über 90 % in einer unlöslich estergebunden Form vorlagen. Der hohe Anteil an freier Zimtsäure im Vergleich zur *p*-Cumar- und Ferulasäure wirkte sich zudem direkt auf die Transferraten vom Malz in die Würze aus, welche für Zimtsäure bei über 100 % und für die erwünschten Phenolsäuren bei kleiner 8 % lag. Ein Vergleich der Gersten- und Weizenmalze hinsichtlich deren Eintrag an Zimt-, *p*-Cumar- und Ferulasäure in die Würzen zeigte, dass die Verwendung von Gerstenmalzen zu einem Eintrag in einem Verhältnis von 2:14:84 and von Weizenmalzen von 1:2:97 führt. Als nächstes, mit dem Ziel eines besseren Verständnisses der Enzymaktivitäten, welche direkt oder indirekt an der Freisetzung der Phenolsäuren während des Mälzens beteiligt sind, wurden die Korn- und Malzproben aus selbigem Probensatz auf deren α- und β-Amylase-, β-Glucanase-, Feruloylesterase- und Proteaseaktivitäten untersucht. Zusätzlich wurden die totalen und wasserextrahierbaren Arabinoxylan- und β-Glucanegehalte in diesen Proben bestimmt, da in Getreide die Zielanalyten hauptsächlich an das Nicht-Stärkepolysaccharid Arabinoxylan gebunden sind. Anhand eines vollständigen Abbaus des β-Glucans und damit der Zellwände konnte der Einschluss von Phenolsäuren in intakten Zellwänden und damit deren Unzugänglichkeit durch Enzyme ausgeschlossen werden. Wie erwartet zeigte sich eine positive Korrelation zwischen dem Abbau des Arabinoxylans und der Freisetzung der Phenolsäuren. Interessanterweise bestand jedoch kein direkter Zusammenhang zwischen der Aktivität der Feruloylesterase, welche für die direkte Freisetzung der Phenolsäuren verantwortlich ist und der freien Phenolsäurekonzentration, was darauf hinwies, dass die Feruloylesteraseaktivität nicht exklusiv für den Gehalt der freien Phenolsäuren verantwortlich ist und weitere Faktoren wie Inhibitorproteine und die Gesamtgehalte der Phenolsäuren berücksichtigt werden müssen. Die Abwesenheit einer Korrelationen zwischen der Proteaseaktivität und den untersuchten Enzymaktivitäten zeigte zudem, dass die an der Freisetzung der Phenolsäuren beteiligten Enzyme nicht durch die Proteasen angegriffen werden.

Aufbauend auf diesen neuen Erkenntnissen wurden im zweiten Teil dieser Arbeit ausgewählte Mälzungsparameter, wie die Weichtemperatur, die Keimtemperatur in Kombination mit der Belüftungsrate und die Temperaturen, welche während des Darrens verwendet werden, auf deren Eignung zur Styrolreduktion untersucht. Für jeden dieser Parameter wurden mehrere Mälzungsexperimente, unter Einsatz des selben Rohmaterials bestehend aus zwei Gerstensorten und einer Weizensorte, durchgeführt. Aus den Malzen wurden im Weiteren Würzen hergestellt, um festzustellen, inwiefern sich der Mälzungsprozess auf die Präkursorenkonzentrationen in den Würzen auswirkt. Das größte Potential zur Styrolreduktion zeigte die Schwelktemperatur. Niedrigere Temperaturen sollten hier bei der Mälzung von Gerste und Weizen höheren Temperaturen vorgezogen werden. Im Labormaßstab ließ sich durch die Auswahl niedrigerer Schwelktemperaturen eine Reduktion der Styrolkonzentration von bis zu 72 % erreichen. Der zweitgrößte Einfluss zeigte die Keimtemperatur zusammen mit der Belüftungsrate, wobei in diesem Fall höhere Temperaturen und niedrigere Belüftungsraten sich vorteilig auf eine Reduk-

tion von Styrol mit einem Reduktionspotential von bis zu 52 % auswirkten. Den geringsten Einfluss zeigte die Weichtemperatur, mit der Fähigkeit durch Einsatz höherer Temperaturen die Styrolkonzentration um bis zu 16 % zu senken. Interessanterweise zeigte der Mälzungsparameter mit der höchsten Prozesstemperatur auch den größten Einfluss, was vermutlich auf die Temperaturabhängigkeit der Enzyme zurückzuführen ist.
Bisher wurden die Gersten- und Weizensorten immer getrennt untersucht, weshalb im dritten Teil dieser Arbeit Würzen unter Einsatz von unterschiedlichen Verhältnissen von Gersten- zu Weizenmalz hergestellt wurden. Die Daten aus diesen Experimenten dienten dazu, zu bestimmen, ob und wie sich die Phenolsäurekonzentrationen und Enzymaktivitäten in diesen beiden Getreidesorten gegenseitig beeinflussen, wenn diese bei der Weizenbierherstellung miteinander verschnitten werden. Dabei zeigte sich, dass es zu keinen synergistischen Effekten kommt, wenn Gersten- und Weizenmalze bei der Herstellung von Weizenbier vermengt werden. Stattdessen wurde eine lineare Korrelation in Abhängigkeit vom Verhältnis zwischen Gersten- und Weizenmalz und den Phenolsäurekonzentrationen festgestellt. Der Gehalt an Phenolsäuren in Würzen zur Weizenbierherstellung ist demnach eine Funktion der Konzentration in Würzen aus 100 % Gerste oder Weizen, multipliziert mit dem jeweiligen Prozentsatz der für das Einmaischen verwendeten Getreideart. Abschließend wurden die Würzen, im Hinblick auf die hefeeigene Feruloylesteraseaktivität und der fehlenden Korrelation zwischen den freien Phenolsäurekonzentrationen in den Prozessintermediaten zu den Vinylaromatkonzentrationen in Weizenbier, in Gegenwart von zwei unterschiedlichen Pof-aktiven, obergärigen Hefestämmen fermentiert. Dadurch sollte die Frage geklärt werden, zu welchem Anteil die löslich estergebundenen Phenolsäuren zur finalen Vinylaromatkonzentration in Weizenbier beitragen. Vergleichbare Konzentrationen der löslich estergebundenen Phenolsäuren in Würze und Bier zeigten dabei, dass es zu keiner Freisetzung löslich estergebundener Phenolsäuren und damit im folgenden zu einer Umsetzung zu den Vinylaromaten gekommen ist. Gleichzeitig wurde ein vollständiger Abbau der freien Phenolsäuren durch die Fermentation beobachtet, wodurch abschließend gezeigt werden konnte, dass die finalen Vinylaromatkonzentrationen hauptsächlich durch die freien Phenolsäurekonzentrationen in den Ausschlagswürzen und weniger durch die Gehalte der löslich estergebundenen Phenolsäuren oder der hefeeigenen Feruloylesterase bestimmt werden.

Contents

1 Introduction

The occurrence of toxicologically relevant compounds in foods is often associated with contamination from outside, e.g. from food packaging; however, foods themselves can also be a major source of toxicants. This is the case for wheat beer, where styrene (S) is found as a natural component. According to the carcinogenic toxicity of styrene, mitigation strategies were in the focus of research, with the aim to reduce the exposure of consumers to styrene by drinking wheat beer. Therefore, each step in the brewing process was examined for its suitability for styrene mitigation. The influence of the process steps of mashing, wort boiling, and fermentation on the styrene concentration was already subject of the work of Langos et al. and Schwarz et al. Lining up with these previous studies, this thesis investigated the impact of the various malting parameters on the release of the precursors of desired and undesired vinyl aromatics and how this affected the final concentrations of the vinyl aromatics in wheat beer.

1.1 Wheat Beer

Wheat beer is a unique beer type, well known to the German speaking areas of Europe, Germany and Austria. Within the German purity law, wheat beer is a special candidate, as it is the only exception where an additional ingredient, namely wheat malt, is allowed beside barley malt, hop, water, and yeast. Thereby, by law, the wheat malt content in wheat beer has to be at least 50%, in order to be classified as wheat beer. An additional characteristic of wheat beer is the use of top fermenting yeasts during fermentation, which has a decisive effect on the overall aroma. Different styles of wheat beer exist on the market. A "classical" wheat beer or "Hefeweizen" has a pale colour and shows a natural turbidity, as it is produced from pale malts without removing the yeast before bottling. If dark barley and wheat malts are used in the brewing process, the output is a so-called "dark wheat beer". If the yeast is removed prior to bottling via filtration, then the wheat beer is called "Kristallklar". Also the reduced-alcohol and nonalcoholic forms of these wheat beer types are found on the market, which enjoy also great popularity. This can be explained by the fact that reduced-alcohol and nonalcoholic wheat beers elicit an aroma, that is very close to a classical wheat beer, rather than to the aroma of wort, which is normally found for other reduced-alcohol and nonalcoholic beer types.
With an annual production volume of approximately 9 and 0.2 million hL in Germany and Austria in 2018, wheat beer made up 9.2 [1] and 2.0% [2], respectively, of the annual aggregated

beer output in these countries. According to a market survey from 2019, wheat beer was the second most popular beer type in Germany [3]. This fact highlights its economical importance and further explains the interest of German breweries into the topic of styrene mitigation along with the maintenance of the desired aroma expected by the consumers.

1.2 The Aroma of Wheat Beer

The wheat beer aroma with its characteristic clove-like and slightly phenolic odour notes clearly differs from the aromas of all other beer types available on the market. In total, over 600 volatile compounds have been reported in different kind of beers [4], however, not all of them are odour-active or are present at concentrations high enough to contribute to the aroma of beer. Langos et al. [5] used the sensomics approach, developed by Grosch [6] and Schieberle [7] (see Section 1.2.1), in order to identify the aroma-active compounds responsible for the wheat beer aroma. Twenty-eight odorants (see Table 1.1) were found to have an odour-activity value (OAV) $\geq$1, which highlights their contribution to the overall aroma of wheat beer. This was verified by the comparison of an aroma recombinate in acidified and carbonized tap water to the corresponding wheat beer in a descriptive profile test. Additionally, Langos et al. compared a wheat beer with a strongly pronounced, typical wheat beer aroma to a wheat beer with an atypical aroma, which was the consequence of the use of a yeast strain differing from the normally used standard wheat beer yeast strain (W68) during fermentation. Interestingly, only two compounds showed strong differences in their OAVs, revealing that these two are in charge of the formation of the distinct wheat beer aroma, namely 2-methoxy-4-vinylphenol (2M4VP) and 4-vinylphenol (4VP) [5]. 2M4VP contributes to a clove-like and smoky odour impression and 4VP to an almond shell-like and phenolic smell. If these two compounds are absent or present in too low concentrations, the corresponding wheat beer exhibits a less pronounced, atypical aroma. According to Wackerbauer et al. [8], wheat beers elicit the typical wheat beer aroma only if the 2M4VP concentration is >600 μg/L.

Table 1.1: Concentrations, odour thresholds, and OAVs of relevant odorants in wheat beer (according to Langos et al. [5]).

Odorant	Concentrations[a] [μg/L]	Odour thresholds[b] [μg/L water]	OAVs
Ethanol	40,100,000	24,900	1,610
(E)-β-Damascenone	1.3	0.004	325
3-Methylbutyl acetate	4,390	19	231
Ethyl methylpropanoate	4.5	0.02	225
Ethyl butanoate	115	1	115
Acetaldehyde	1,720	25	69
3-Methyl-1-butanol	583,00	1,000	58
Dimethyl sulfide	49.6	1	50
3-Methylbutanal	18.8	0.4	47
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	1,110	25	44
Ethyl hexanoate	129	5	29
2-Phenylethanol	21,100	1,000	21
2-Methoxy-4-vinylphenol	2,020	100	20
Linalool	2.8	0.14	20
3-(Methylthio)-1-propanol	4,490	250	18
2-Aminoacetophenone	2.41	0.2	13
4-Vinylphenol	882	78	11
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	1.8	0.3	6
Vanillin	21.6	4.9	5
Methyl-1-propanol	23,100	8,300	3
Ethyl octanoate	220	70	3
Acetic acid	275,000	180,000	2
2-Phenylethyl acetate	518	356	2
γ-Nonalactone	42.4	27	2
3-(Methylthio)propanal	3.1	1.8	2
Butanoic acid	1,180	1,000	1
3-Methylbutanoic acid	794	740	1
1,1-Diethoxyethane	23.2	25	1

[a] Mean values of triplicates. [b] OAVs were calculated by dividing the concentration by the respective odour threshold.

1.2.1 Evaluation of the Aroma Relevance of Volatile Compounds: the Sensomics Approach

The sensomics approach is a concept developed by Grosch [6] and Schieberle [7] to evaluate the aroma relevance of volatile compounds in foods. It contains 6 steps, combining sensory and instrumental methods to identify and quantitate important aroma-active substances, so-called key odorants, in foods:

1. Gas chromatography-olfactometry (GC-O) of an aroma distillate:
   The identification of odorants in foods requires a sample preparation procedure, which allows the extraction of analytes without the formation of any artefacts. Therefore, in flavour science, classically a liquid-extraction is combined with a high vacuum distillation (10^{-4} bar) using the so-called solvent assisted flavour evaporation (SAFE) technique [9], where volatile compounds are separated from non-volatile compounds at low temperatures (40 °C). In order to guarantee low detection limits, the distillates are further concentrated using a Vigreux-column, prior to the analysis by GC-O. GC-O combines the ability of gas chromatography to separate volatile compounds with the detection power of the human nose. During GC-O the gas flow is split (1:1) at the end of the column, which allows the detection of the analytes with the flame ionization detector (FID) and at a heated sniffing-port by the human nose in parallel to evaluate the respective odour activities. Thus, GC-O analysis allows to determine which of the peaks in the chromatogram show an odour activity and which kind of odour impression they have.

2. Aroma extract dilution analysis (AEDA):
   AEDA is a screening method to estimate the importance of an aroma substance to the overall aroma of a certain type of food. Therefore, the aroma distillate is diluted step by step with the corresponding solvent in a ratio of 1 + 1 (v + v). Subsequently, each of the dilution steps is analysed by means of GC-O. The highest dilution, at which an aroma substance still leads to a stimulation of the olfactory system, corresponds to the flavour dilution (FD) factor of this compound. As a consequence, it is supposed that the higher the FD factor of an odorant is the more important is its role for the overall aroma.

3. Identification experiments:
   Different criteria are used to verify the identity of an aroma compound: the odour quality and intensity, the retention indices (RIs) determined on two different columns of different polarities, and the mass spectra generated in the electron ionisation (EI) and chemical ionisation (CI) mode have to match the data obtained for the corresponding authentic reference compounds.

4. Stable isotope dilution analysis (SIDA):
   To evaluate the relevance of an odorant for the overall aroma, its concentration in the food sample has to be known. Therefore, quantitations of the aroma compounds with the highest FD factor, at least an FD factor >1, at maximum 35 compounds are performed. For that purpose, SIDA has been established in flavour science as the most accurate quantitation procedure. This technique uses stable isotopically labelled compounds (isotopologues) as internal standards, which differ from the analytes only in the presence of stable isotopes (classically ^{2}H and ^{13}C) in the molecules. Known concentrations of these stable isotopically labelled compounds are added to the food sample prior to the work-up procedure. According to the nearly identical chemical, physical, and chromatographic behaviour of the isotopologs, losses occurring during work-up can be compensated. Consequently, the ratio of the internal standard and the analyte at the beginning of the work-up corresponds to the ratio after sample preparation. By means of mass spectrometry, analytes and stable isotopically labelled internal standards can be measured separately, due to their mass differences. Now, from the known concentrations of the stable isotopically labelled internal standards and the response curves, the accurate concentrations of the analytes can be determined [10].

5. Calculation of the odour activity value (OAV):
   The concentration itself is not enough to evaluate the role of an odorant on the overall aroma of a certain food, as each aroma substance has its characteristic odour threshold in a distinct matrix. In order to take both the concentration and the odour threshold into account, the OAV was established by Rothe et al. [11]. The OAV is defined as the quotient of the concentration and the substance specific odour threshold, and thus, allows the prediction of the relevance of an odorant to the overall aroma. Per definition, aroma compounds with an OAV $\geq$1 are contributing to the overall aroma.

6. Aroma simulation:
   To verify the qualitative and quantitative results, the aroma of a food sample is simulated and compared to the aroma of the original food in a sensory test. For that purpose, a recombinate is prepared, which consists of a matrix that is most similar to the food sample, but lacks the analytes of interest. To this matrix, the aroma compounds with an OAV $\geq$1 are added in the same concentrations as analysed in the the food sample. If the key odorants have been correctly identified and quantitated, the aroma of the recombinate and the food sample should be similar. Additionally, recombinates including omission experiments allow to study additive and suppressing effects of single odorants, which is not possible by a single consideration of the OAVs.

1.3 Styrene in Wheat Beer

Styrene belongs to the class of aromatic hydrocarbons and shows therefore an apolar, lipophilic behaviour. At room temperature it, is a colourless liquid (bp. 145 °C) with a sweet, solvent- and plastic-like smell. Styrene is best known for its use as a raw material in plastic production, as it is the monomer of polystyrene (PS). This type of plastic is widely used as a packaging material in food industry. Among all food-contact packaging polymers, it is the second most used one [12]. As a consequence of the direct contact to food, migration of the monomer into the foodstuff has to be questioned. Multiple studies [13–15] revealed that the migration of the styrene monomer from PS into food is a serious issue, which has to kept in mind while using PS in food packaging. However, styrene was also found in foods, where migration from food packaging could be excluded. Different studies [16, 17] determined the styrene contents in foods, where a contact to plastic as packaging material was strictly avoided. Therefore, reported concentrations of styrene in these studies could be ascribed to a natural origin. Steele et al. [16], for example, found styrene in wheat, peanuts, coffee, peach, oat, strawberries, and beef and Fragnière et al. [17] proved the presence of styrene in cinnamon. While styrene contents in foods mostly showed relatively low contents (0.23–7.85 μg/kg), cinnamon with very high amounts of up to 40,000 μg/kg is an exception. Another food which contains styrene as a natural component is wheat beer. The presence of styrene in wheat beer, its origin, its toxicological relevance, and how it is linked to the desired vinyl aromatics in wheat beer will be discussed in the following chapter.

1.3.1 First Evidence of Styrene in Beer

It was in the 1970s, when the presence of styrene in beer was attested the first time. Renner et al. [18] analysed 7 different types of beer ("Pils", "Märzen", "Dunkler Bock", "Alt", "Weizen", "Berliner Weiße", and "Doppelhopfen") for their volatile constituents. In 3 out of 7 of these beer types ("Märzen", "Weizen", and "Berliner Weiße"), the presence of styrene was proven with concentrations between 70–160 μg/L. At the same time, Tressl et al. [19] also found styrene in a "Pilsener" beer, where a content of 70 μg/L was detected. No further attention was paid to the issue of styrene in beer until 1997 when Daly et al. [20] reviewed the topic, questioning the origin of styrene (see Section 1.3.3) with a special focus on wheat beer. The motivation behind this new study was the previous declaration of styrene as a toxicologically relevant compound in 1996 by the World Health Organisation (WHO) (see Section 1.3.2). This study was later on followed by the studies of Schwarz et al. [21–25], Langos et al. [5, 26–29], and Kalb et al. [30–34] starting in the year of 2011, who investigated the effect of the different steps of the brewing process on the release of styrene, with the aim to find mitigation strategies to reduce the styrene content in wheat beer (see Section 1.5). Daly et al. [20], Langos et al. [28], and Kalb et al. [30] examined independently of each other in market surveys the styrene content in wheat beers (see Table 1.2). Thereby, the styrene contents in classical wheat beers were

between 9.8–33 μg/L, which was significantly lower than the concentrations found by Renner et al. [18] and Tressl et al. [19]. Additionally, none of these studies could confirm the presence of styrene in bottom-fermented beers such as "Pils" and "Märzen". A possible explanation was found in the results of Renner et al., who also analysed the effect of pasteurisation, sunlight, UV-light, and storage time (2 years) on a "Pilsner" beer. Only in case of pasteurisation, relevant styrene contents were found. Therefore, a thermal decarboxylation in case of the analysed "Pils" and "Märzen" samples could be the explanation for the found styrene contents.

Table 1.2: Concentration ranges of styrene (S), 4-vinylphenol (4VP), and 2-methoxy-4-vinylphenol (2M4VP) in wheat beer.

			Concentration ranges [μg/L] of			
Literature	Fermentation	Pof-activity	S	4VP	2M4VP	n[a]
Langos et al. 2016 [28]	Top, pale	+	15–33	620–1,020	630–2,020	6
	Top, r.-alc.[b]	+	25	355	795	1
Daly et al. 1997 [20]	Top, pale	+	25–31	-	-	4
	Top, r.-alc.	+	9, 25	-	-	2
Wackerbauer et al. 1982 [8]	Top, pale	+	-	440–2,700	520–4,300	21
Kalb et al. 2019 [30]	Top, pale	+	9.8–32	630–1,350	1,100–2,500	18
	Top, r.-alc.	+	13	650	900	1
	Top, alc. free[c]	+	14	510	370	1
	Top, dark	+	12	430	620	1
	Top, pale	+/–	0.2	65	140	1
	Top, dark	+/–	0.7	56	110	1
	Top, r.-alc.	+/–	1.8	32	61	1
	Top, alc. free	+/–	0.6	40	78	1

[a] Number of samples. [b] Reduced-alcohol. [b] Nonalcoholic.

The reason for the found differences in the styrene concentrations in these studies might be beside different samples the use of different analytical techniques including the type of standards used for quantitation. Renner et al. [18] used a special glass equipment developed for the dynamic extraction of volatile compounds prior to GC analysis. Briefly, nitrogen was purged into the beer sample, which was heated on a water bath. Thereby, volatile compounds were stripped off the solution and trapped on a column filled with Propak as adsorption material. The trapped analytes were thermally desorbed and collected on a cold trap, consisting of a special glassware that was cooled with dry ice. Methyl decanoate, methyl heptanoate, and methyl butanoate were used as internal standards and added to the beer samples prior to the dynamic extraction. The idea behind an internal standard is to simulate the chemical and physical behavior of the analyte of interest as best as possible. For sure, the selected standards are not the best choice for styrene, due to the significant structural differences, e.g., the presence

of an ester moiety and the lack of an aromatic ring. In contrast, Tressl et al. [19] applied a liquid-liquid extraction with a mixture of *n*-pentane and dichloromethane (2:1, v:v) as the extraction solvent for the isolation of volatile compounds from beer. Compared to the other studies, no internal standard was used, which was considered by the authors with approximately results. The applied technique for the analysis of styrene of Daly et al. [20] was an advancement of the dynamic extraction technique of Renner et al. [18], the so-called dynamic headspace (DHS) technique, using Tenax TA as adsorbent in combination with a thermal desorption unit (TDU) connected with the GC system. For the correction of analyte losses, methyl propyl ketone was used as internal standard, which can again only be an approximation of the true value due to the structural difference to styrene. Therefore, the most important difference between the recent studies by Langos et al. [28] and Kalb et al. [30] to the above described methods is given by the application of SIDA for the quantitation of styrene, allowing maximum correction by the use of a stable isotopically labelled analog of the analytes of interest. For sample preparation, Langos et al. used a combination of liquid-liquid extraction (diethyl ether) with SAFE distillation, whereas Kalb et al. applied the headspace-solid phase microextraction (HS-SPME) technique.

1.3.2 Toxicological Relevance of Styrene

Although the presence of styrene in wheat beer was known since the 1970s, its toxicological relevance was not considered until 1996, when the WHO published a tolerable daily intake (TDI) for styrene of 7.7 μg/kg body weight per day [35]. At that point, styrene was not a major concern for brewers, as an average person with 70 kg would have to consume >18 L of wheat beer with a styrene concentration of 30 μg/L (cf. Table 1.2) per day to exceed this limit. However, in 2002, styrene was classified by the International Agency for Research on Cancer (IARC) into "group 2B", as "possibly carcinogenic to humans" [36]. Since 2006, also the Federal Institute of Risk Management (Bundesinstitut für Risikobewertung, BfR) lists styrene according to the classification made by the IARC as a carcinogen [37]. The problem with carcinogenic compounds is that due to the additive effects with every contact the probability to get cancer increases. Therefore, styrene got a major issue in wheat beer and minimisation strategies had to be found to reduce the exposure of consumers to styrene by drinking wheat beer.
Brewers with beer exports to the US also have to be aware that styrene is listed in the "Proposition 65" [38] since 2016. Proposition 65, also called "the safe drinking water and toxic enforcement act", is a Californian law passed in 1986, with the aim to protect drinking water sources from cancer and birth defect causing toxicants. However, its validity affects also consumer products, as in general the exposure to those chemicals should be reduced or eliminated. Thus, products must be labelled with a clear and reasonable warning, if one of these toxicants are present to properly inform consumers. For styrene, a non-significant risk level (NSRL) of 27 μg per day is mentioned in the Proposition 65. Therefore, in dependence of the styrene concentration in wheat beer (cf. Table 1.2), already 1 L of wheat beer might exceed

this limit. As a consequence, a serious evaluation has to be done to check whether wheat beer is affected by this law.

The classification of styrene as possibly carcinogenic to humans followed the results of two studies on mice [39] and rats [40]. However, only in case of mice, the inhalation of styrene led to the formation of lung tumours. Interestingly, styrene itself is not toxic, just the metabolisation to styrene oxide triggers the tumour formation. The reason is the formed epoxide, which is highly reactive and tends to bind to DNA. Styrene oxide is built from styrene in presence of two enzymes of the class of the cytochrome P450 (CYP) family (Figure 1.1) [41]. CYPs are a class of enzymes, which almost exclusively act as mono-oxygenases. They play a very important role in the metabolisation of water-insoluble compounds, whereby the oxidation increases the water solubility, and therefore, accelerates the excretion of these substances. In rats, the formation of styrene oxide was absent upon exposure to styrene, which could be attributed to the absence of the corresponding CYP enzymes and explained why no tumours were formed in rats. For humans, opposite results were found [42–47], regarding the presence of these CYP enzymes in the human lung. A resent study by the BfR [37] indicated the presence; however, the question, if the corresponding concentrations of the enzymes are high enough to generate toxicologicaly relevant amounts of styrene oxide is still open [48].

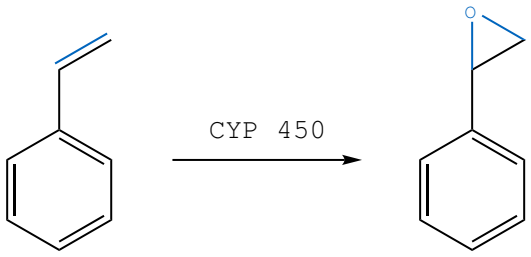


Figure 1.1: Metabolic fate of styrene. The carcinogenic toxicity of styrene is a result of the conversion to styrene-7,8-oxide by the metabolism in the stressed organism by enzymes of the cytochrome P450 (CYP) family.

1.3.3 Link between Undesired, Toxicologically Relevant Styrene and the Desired Odour-Active Vinyl Aromatics in Wheat Beer

A comparison of the styrene molecule with the key odorants in wheat beer reveals their high structural similarity. In fact, this chemical relationship is a consequence of the formation of these vinyl aromatics from precursors that belong to the same compound class of cinnamic acid derivatives, traditionally referred to as phenolic acids [49]. The precursors of interest are cinnamic acid (C), *p*-coumaric acid (pC), and ferulic acid (F), which lead specifically to the formation of styrene, 4VP, and 2M4VP (Figure 1.2). This context already gives a hint to the difficulty of the objective of this project to reduce the styrene content in wheat beer without weakening or eliminating the characteristic wheat beer aroma expected by the consumers.

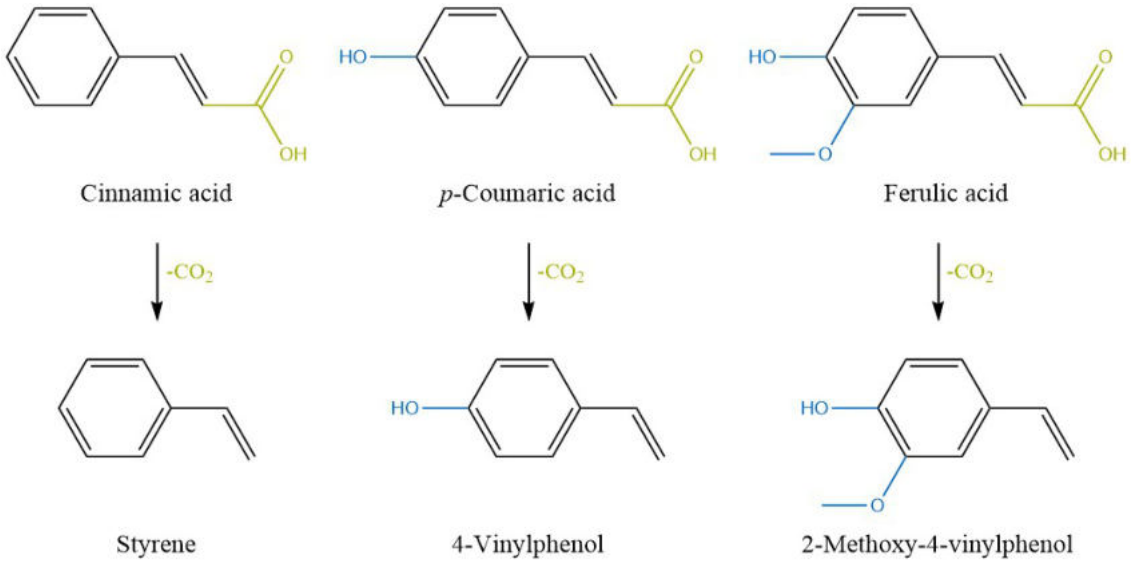
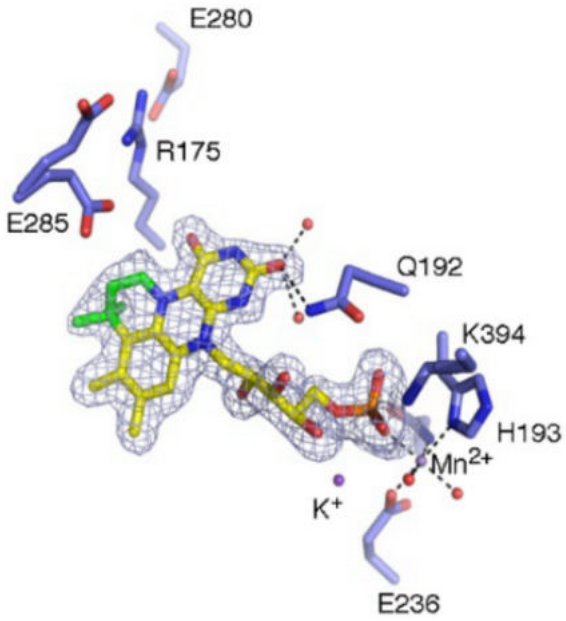


Figure 1.2: Assignment of the precursors cinnamic, *p*-coumaric, and ferulic acid to the corresponding vinyl aromatics styrene, 4VP, and 2M4VP. Decarboxylation of the phenolic acids to the corresponding vinyl aromatics within the brewing process can be either thermally of enzymatically induced.

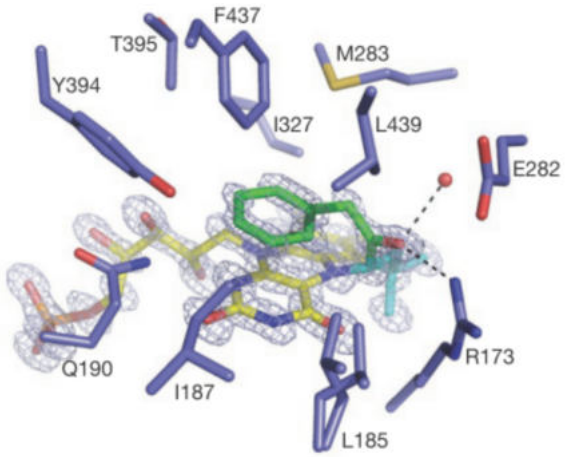
Except for wheat beer, these vinyl aromatics lead to an undesired off-flavour in other beer types, the so-called phenolic off-flavour (Pof). Early investigations on the formation of these vinyl aromatics were therefore motivated by the effort to prevent this off-flavour formation in beer. The mechanism, responsible for the transformation of the precursors into the corresponding vinyl aromatics, is a so-called decarboxylation process, where the carboxylic acid function is eliminated via the release of carbon dioxide. This elimination can be induced either thermally or enzymatically.

The first evidence for an enzymatic induced formation of the vinyl aromatics was given by the study of Wackerbauer et al. [50] in 1978. A sterilised cast wort was inoculated with an isolated yeast from an industrial-scale fermentation that led to a beer with a phenolic off-flavour and

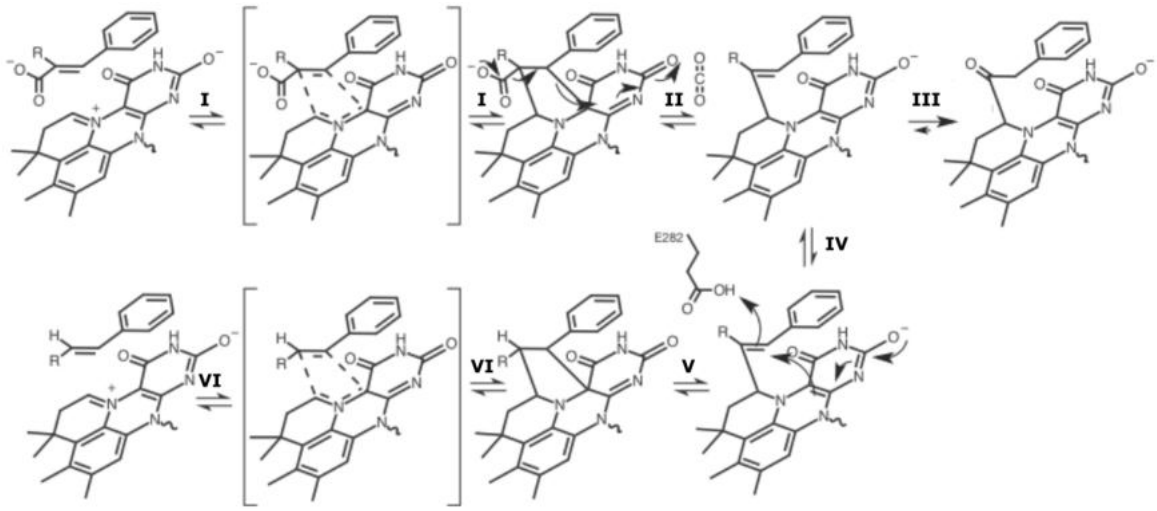
to the formation of 4VP and 2M4VP. An investigation of the used brewing yeast revealed a contamination by a wild yeast strain, which belonged to the yeast family of *Saccharomyces cerevisiae*. A repetition of the fermentation experiment, once with the addition of *p*-coumaric acid and once with ferulic acid, proved the theory of the conversion of these phenolic acids in presence of the yeast. Additionally, 95 brewing yeast strains were tested for their ability to decarboxylate phenolic acids; thereby, bottom-fermenting strains were unable to form vinyl aromatics, while in the group of top-fermenting yeasts, both forming and non-forming strains were found. Daly et al. [20] further demonstrated that different top-fermenting yeast strains express the decarboxylation activity with different intensities. This phenomena was ascribed to Pof^{+}, $Pof^{+/-}$, and Pof^{-} in accordance with a high, medium, or absent Pof activity. Goodey et al. [51] assigned the decarboxylation ability to the presence of the single dominant phenylacrylic acid decarboxylase (POF1) gene in yeasts. Additionally, a test with 11 substrates revealed that the *S. cerevisiae* strain BRG 520 was only able to convert cinnamic, *p*-coumaric, and ferulic acid, whereas, e.g., caffeic acid was not converted. Interestingly, the POF1 gene is highly conserved in all yeast strains, independent of their ability to convert phenolic acids [52]. Very small differences in the sequence of the POF1 gene were found between yeast strains with and without a decarboxylation activity, which refers to a control of the POF1 gene on a very precise level [53]. Clausen et al. [54] were the first, who were able to clone and sequence the total POF1 gene isolated from *S. cerevisiae*. The introduction of the POF1 gene into a Pof inactive yeast resulted in a recovery of the Pof activity. A recent study by Mukai et al. [55] revealed that an additional gene, the so-called ferulic acid decarboxylase (FDC1) gene, is involved in the decarboxylation process. A first hypothesis assumed that both enzymes, the POF1 and the FDC1, encoded by these genes, form an enzymatic complex and function as a phenylacrylic acid decarboxylase. This theory was disproved by a study of Payne et al. [56], as no direct interaction could be detected between the purified proteins from FDC1 and POF1. Instead, they could prove that FDC1 is solely responsible for the decarboxylation activity; however, a cofactor is needed. The role of POF1 for the decarboxylation ability could further be referred to the synthesis of this cofactor, which was identified as a prenylated flavin mononucleotide (prFMN). The found structure of a covalent inhibitor-cofactor adduct, as the product of the exposure of the enzymatic system to inhibitor proteins, unravelled the decarboxylation mechanism as a 1,3-dipolar cycloaddition.



(a) Detailed view of the FDC1 active site of *S. cerevisiae*.



(b) Complexation of phenylpyruvate in the FDC1 active site.



(c) Proposed mechanism of the α,β-unsaturated decarboxylation via 1,3-dipolar cycloaddition of cinnamic acid derivatives.

Figure 1.3: Insight into the FDC1 mechanism. 1.3a, Active site of FDC1 of *S. cerevisiae* with the cofactor prFMN in complex with Mn^{2+} [56]. **1.3b**, Complexation of a phenylpyruvate derived adduct in the active site of FDC1 [56]. **1.3c**, Proposed mechanism [56] for the catalysis of the decarboxylation of phenolic acids (R=H, cinnamic acid) by FDC1 with the following steps: **I** 1,3-dipolar cycloaddition of the phenolic acid to the prFMN-cofactor accompanied by the formation of a pyrrolidine adduct, **II** Grob-type decarboxylative fragmentation, **III** keto-enol tautomerisation, **IV** exchange of CO_2 by the amino acid E282 (glutamic acid), **V** formation of a second pyrrolidine adduct induced by the protonation by E282, and **VI** *retro* 1,3-dipolar cycloaddition and release of the corresponding decarboxylation product.

With regard to the thermal decarboxylation of phenolic acids, Tressl et al. [57] studied the thermal degradation of ferulic acid at 150 °C, proving the formation of 2M4VP. Further, Samaras et al. [58] examined the correlation between the kilning temperature and the amount of free *p*-coumaric and ferulic acid in barley malts. With increasing temperatures, a massive decrease of free phenolic acids was found, with an increase of the concentration of 2M4VP in parallel. Thereby, it was shown that a thermal decarboxylation did not occur at temperatures <140 °C on the one hand, and that temperatures >220 °C already led to a massive generation of 2M4VP on the other hand. These results indicated that thermal processing steps during the production of pale wheat beer should have no effect on the formation of the vinyl aromatics, as pale malts are kilned at temperatures of about 80 °C and temperatures during mashing and wort-boiling are <100 °C. This finding was later confirmed by Langos et al. [28], who monitored the free phenolic acid contents as well as the vinyl aromatic concentrations along the whole brewing process. Thereby, very low vinyl aromatic and very high free phenolic acid contents were found in unboiled wort and cast wort, with the low concentrations of the vinyl aromatics in these process steps originating from thermal decarboxylation during kiln-drying, mashing, and wort boiling. However, the most significant and strongest increase of the vinyl aromatics together with a nearly complete degradation of the free form of the precursors was found during fermentation, thus, highlighting the predominant formation of vinyl aromatics via an enzymatic decarboxylation in the presence of yeast.

1.4 Phenolic Acids

In plant metabolomics, the term "phenolic acids" describes a distinct group of naturally occurring organic compounds, whose structures all derive from the basic sceleton of cinnamic acid [49]. Derivatives of this compound class differ in the degree of hydroxylation and the positions of the hydroxyl groups at the aromatic ring. Although cinnamic acid lacks a hydroxyl group at the aromatic ring, it is part of the group of phenolic acids, due to the biological relationships.

1.4.1 Biochemical Formation of Phenolic Acids

The biochemical formation of phenolic acids (see Table 1.4), the so-called phenylpropanoid metabolism [59, 60], starts from the aromatic amino acids phenylalanine and tyrosine, both products of the shikimate pathway [61]. In the first step, these two amino acids are deaminated either by the enzyme phenylalanine ammonia-lyase (EC 4.3.1.24) or the enzyme tyrosine ammonia-lyase (EC 4.3.1.25). This leads to the formation of cinnamic acid from the precursor phenylalanine and to *p*-coumaric acid from the precursor tyrosine. Cinnamic acid, however, is further transformed to *p*-coumaric acid via hydroxylation at the 4′-position in the aromatic ring. The responsible enzyme is the cinnamate 4-hydroxylase (EC 1.14.13.11). Therefore, cinnamic acid plays only the role of an intermediate in the phenylpropanoid metabolism. In contrast,

p-coumaric acid is the starting point for two biosynthesis pathways, the lignin biosynthesis pathway and the flavanoid biosynthesis pathway. In the lignin pathway, *p*-coumaric acid is subject to further hydroxylation and methylation steps, leading to the formation of ferulic acid and sinapinic acid. These three phenolic acids, *p*-coumaric, ferulic, and sinapinic acid, now serve as the basic building blocks for the biosynthesis of lignin. From a quantitative perspective, lignin is the most important phenolic compound in plants, where it plays an important role as a key structural material. In the flavanoid pathway, *p*-coumaric acid is esterified to the coenzyme A (CoA) by the action of the enzyme 4-coumarate-CoA ligase (EC 6.2.1.12). The corresponding *p*-coumaric acid-CoA is the major building block in the biosynthesis of flavanoids and stilbenoids. These plant secondary metabolites have multiple tasks in plants, where they act as antimicrobials, photoreceptors, visual attractors in form of pigments, UV protectors, feeding repellents, etc. In fact, this broad use of phenolic acids highlights their importance within the plant kingdom and their occurrence in high amounts.

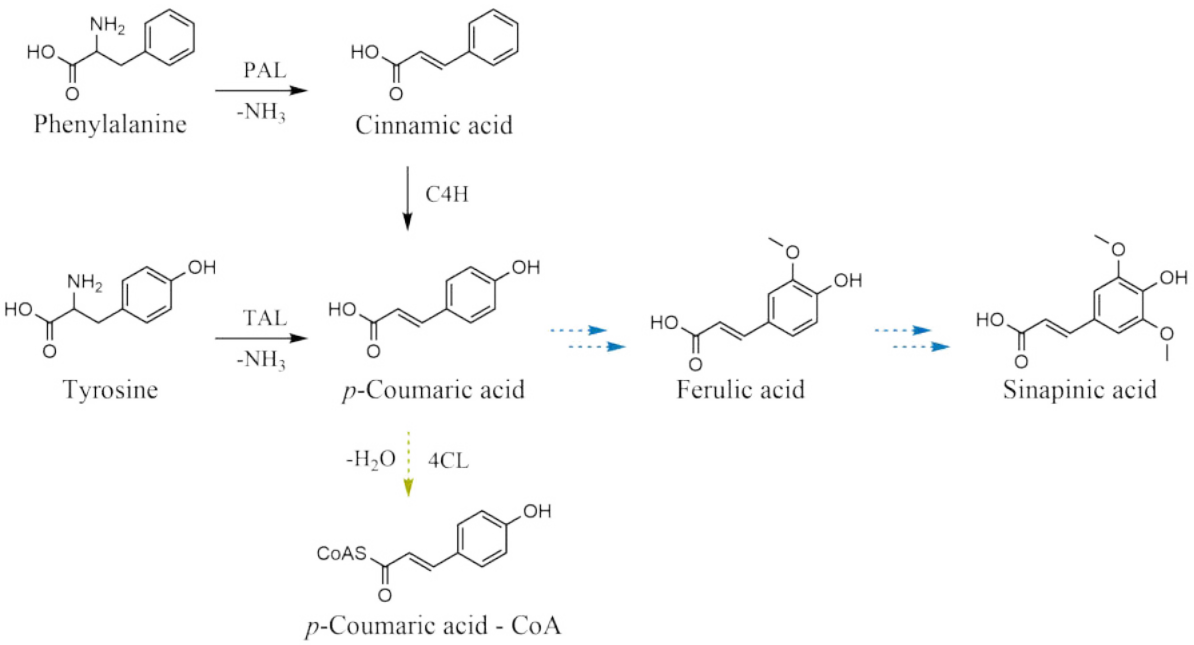


Figure 1.4: Biosynthesis of phenolic acids via the phenylpropanoid metabolism. Starting from phenylalanine and tyrosine, *p*-coumaric acid is either directly formed by deamination or via cinnamic acid, with an additional hydroxylation step. With *p*-coumaric acid, two biosynthesis pathways start, the lignin (in blue) and the flavanoid (in green) pathway. (PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; TAL, tyrosine ammonia-lyase; 4CL, 4-coumarate-CoA ligase)

1.4.2 Introduction of Phenolic Acids into the Brewing Process

Within the brewing process, another structure in terms of phenolic acids is of great interest, the so-called arabinoxylan structure. This nonstarch polysaccharide (NSP) consists of straight sugar chains built up from xylose, with single arabinose sugars attached irregularly [62]. These straight polysaccharide chains are linked to a three-dimensional network via bridges built up from phenolic acids, which are bound to the arabinose moieties [63, 64]. Chemically, this bridging units are covalent bonds formed between the aromatic rings of two phenolic acids of opposing arabinoxylan chains. In cells of the grain of barley and wheat, this arabinoxylan three-dimensional network plays a very important role, as it is used to build up the cell walls. Now, it is exactly this arabinoxylan structure, which is claimed in literature to be the main source of phenolic acids in the brewing process [60, 65, 66]. In fact, it could be shown that the majority of ferulic acid in grain of barley and wheat is esterified to the arabinoxylan structure [67].

Grain of barley and wheat consist of two main parts, the endosperm and the bran fraction, which account for 75 and 25% of the grain, respectively. Within these fractions, arabinoxylans as well as the phenolic acids are unevenly distributed. Again, it could be shown that the content of ferulic acid in wheat bran was around 52–70 times higher than in the endosperm [68]. Moreover, the majority of ferulic acid in the bran could be ascribed to the present aleurone cells [69], which are especially rich in arabinoxylan [70]. Among phenolic acids in barley and wheat, ferulic acid shows by far the highest amounts. A study of Li et al. [71] on 130 winter wheat varieties and 20 spring wheat varieties revealed that ferulic acid accounts in average for 59 and 66%, respectively, of the total phenolic acids in these varieties. In contrast, *p*-coumaric acid made up only 2.3 and 1.8%, respectively, of the overall phenolic acid content. In barley, the situation was shown to be quite similar, with approximately 59 and 13% of the total phenolic acid content in 10 barley varieties were made up by ferulic and *p*-coumaric acid [72]. Interestingly, up to now, the total cinnamic acid content in barley and wheat has never been investigated.

Beside the used malt in the brewing process, also hop could be a source for phenolic acids. However, Wackerbauer et al. [73] proved that hop had no significant contribution to the input of phenolic acids into the brewing process.

1.4.3 Impact of the Brewing Process on Phenolic Acids

In the beginning of the brewing process, arabinoxylan preliminary exists in its intact form in the unmalted barley and wheat. Now, during the first process step (malting), the grain expresses different enzymes to release maltose from starch and amino acids from storage proteins accessible for the seedling. As a consequence of this germination process, cell walls have to be degraded, as for example starch is stored within the endosperm cells. In total, four enzymes are responsible for the degradation of the arabinoxylan. Three of them, namely endo-1,4-β-xylanase (E.C 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), and α-L-arabinofuranosidase (EC 3.2.1.55), degrade

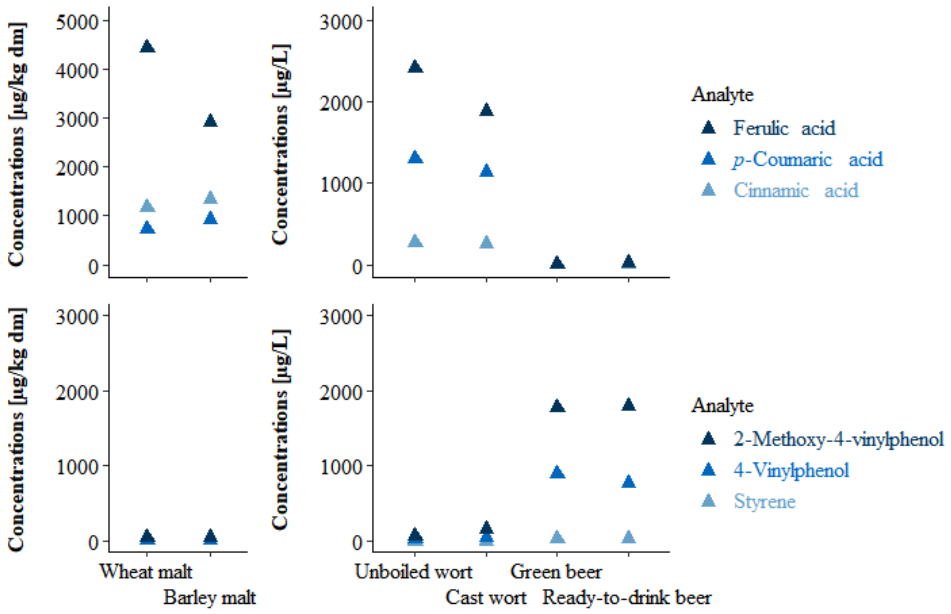
the polysaccharide chain into smaller fragments and an additional one, the feruloyl esterase (FEA, EC 3.1.1.6), directly cleaves the ester bonds between the phenolic acids and the arabinose moieties [74]. This leads to the formation of three different forms of phenolic acids with respect to the size of the fragments and the associated water solubility, the free, soluble ester-bound, and insoluble ester-bound phenolic acids. During the next step of the brewing process (mashing), these enzymes are still active [74, 75] and lead to a further degradation of the arabinoxylan, and therefore, to an increase of the content of the free and soluble ester-bound forms. Additionally, this step is crucial, as now the free and soluble ester-bound phenolic acids from malt are transferred into the wort, determining the maximum amount of phenolic acids which are later accessible for the yeast. Following, within wort boiling, the enzymes of interest are denatured [74]. During the last step of the brewing process (fermentation), solubilised phenolic acids in wort are decarboxylated in the presence of yeast. Interestingly, yeasts have their own feruloyl esterase activity [76], allowing them to release free phenolic acids from the soluble ester-bound form.

1.5 Previous Studies on Styrene Mitigation

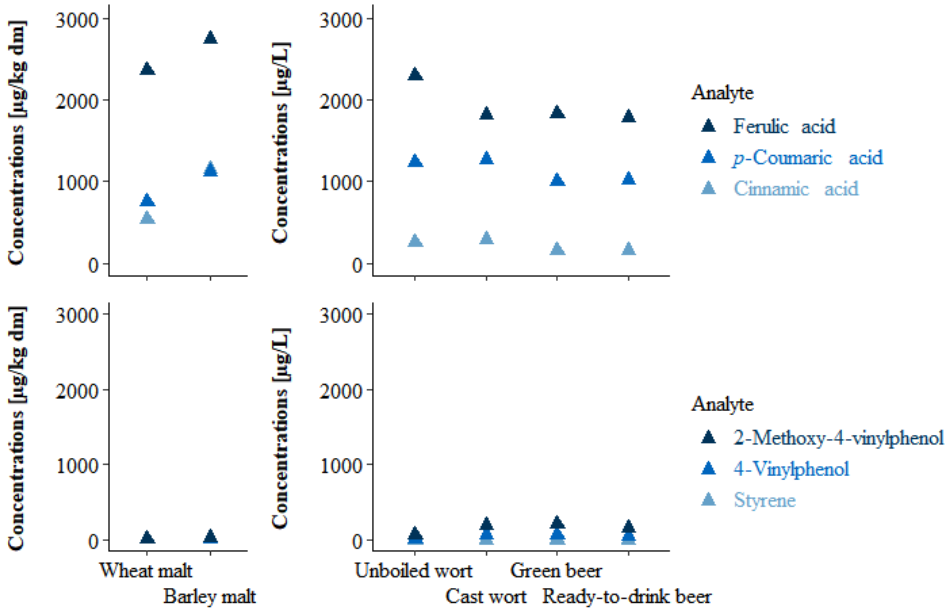
Due to the classification of styrene as "possibly carcinogenic to humans" by the IARC in 2002, the brewing industry got sensitised to its toxicological relevance and as a response, studies on the influence of the different brewing steps on the styrene concentration were performed. This chapter addresses the results of previous studies, with the aim to summarise the knowledge about the impact of the various processing steps on the release of styrene studied so far. In all of these studies, styrene was always discussed with respect to the desired vinyl aromatics, as the preservation of the characteristic wheat beer aroma expected by the consumers had to be guaranteed.

1.5.1 Concentration Trends of the Vinyl Aromatics and Phenolic Acids during Wheat Beer Brewing

In order to determine the contribution of each brewing step to the release of the free phenolic acids and to the formation of the respective vinyl aromatics, Langos et al. [28] investigated the concentrations of the desired and undesired vinyl aromatics as well as of the corresponding free phenolic acids within two industrial-scale brewing experiments A and B (Figure 1.5). The difference between these two experiments was given by the used yeast strain during fermentation, with one yeast strain revealing a high (A) (Figure 1.5a) and the other showing no (B) Pof-activity (Figure 1.5b).



(a) Phenolic acid (top) and vinyl aromatic (bottom) pattern during wheat beer production with a Pof$^+$, top-fermenting yeast.



(b) Phenolic acid (top) and vinyl aromatic (bottom) pattern during wheat beer production with a Pof$^-$, top-fermenting yeast.

Figure 1.5: Concentration courses of free phenolic acids and vinyl aromatics during two industrial-scale brewing processes. 1.5a, Analyte pattern of a brewing process, where a Pof$^+$, top-fermenting yeast was applied during fermentation. **1.5b**, Analyte pattern of a brewing process, where a Pof$^-$, top-fermenting yeast was used. In both cases, a bottom-fermenting yeast was applied for the second fermentation (bottle fermentation). (Raw data from Langos et al. [28].)

1. Mashing:
   A comparison of the found free phenolic acid contents in the wort with the theoretical contents in case of a 100% transfer from malt into wort revealed that additionally to a complete solubilisation, an extensive release of phenolic acids occurred during mashing. Interestingly, the ratio between free cinnamic acid and ferulic and *p*-coumaric acid increased significantly in wort, although, for example, free cinnamic and *p*-coumaric acid showed comparable concentrations in the corresponding malts. This finding can be explained by a much stronger release of the desired phenolic acids or/and by a lower abundance of bound cinnamic acid in malts. Moreover, the two different sets of malts of experiment A and B showed different free phenolic acid patterns; however, they had similar patterns after mashing. This data suggest that there is no correlation between the free phenolic acid contents in malt and wort.
   Already in the malts of A and B, very low amounts of the vinyl aromatics were present, which could be ascribed to a thermal decarboxylation as a consequence of the kilning temperature. Within the mashing process, the contents of 4VP and 2M4VP were slightly higher than the calculated contents based on transfer rates of 100%, suggesting that to some extent, a slight formation of the desired vinyl aromatics occurred. In contrast, styrene contents were lower than the calculated ones, indicating that styrene is probably lost by evaporation during mashing.

2. Wort boiling:
   During wort boiling, a moderate increase of the vinyl aromatics was accompanied with a moderate decrease of the free phenolic acids. The reason for that is given by the presence of a thermal decarboxylation during wort boiling.

3. Main fermentation:
   Within the first fermentation, a massive increase of the vinyl aromatics was found together with a nearly complete degradation of the free phenolic acids in experiment A. In contrast, in experiment B significant changes in the content of the analytes were not observed. This can be explained by the different Pof-activities of the used yeast strain, which was not expressed by the yeast used in experiment B. A comparison of the vinyl aromatic contents produced by an enzymatic decarboxylation and the amounts formed by a thermal decarboxylation clearly demonstrated the predominant formation via the enzymatic pathway.

4. Second fermentation:
   For the second fermentation, a bottom-fermenting yeast was added to the green beer of both experiments. Neither in experiment A nor in B an increase of the concentrations of the vinyl aromatics was found. This might be explained by the fact that bottom-

fermenting yeasts lack without any exception the ability to decarboxylate phenolic acids, and additionally, with the low amounts of free phenolic acids present in green beer from experiment A. An additional study performed by Schwarz et al. revealed [21] that no correlation was given between the cinnamic acid content in the unboiled wort and the styrene concentration in the ready-to-drink beer.

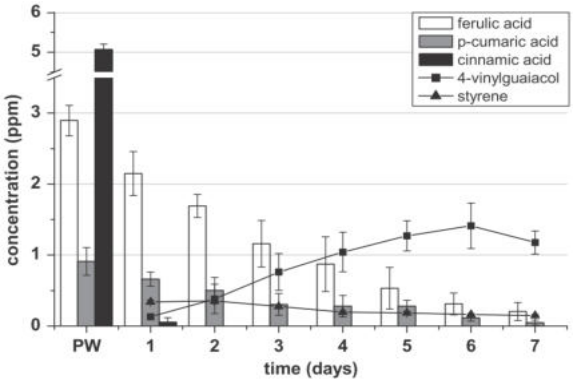
5. Flash pasteurisation:
   Schwarz et al. [21] proved that also flash pasteurisation, capable of a thermal decarboxylation, did not significantly contribute to the final vinyl aromatic contents.

In summary, decarboxylation of phenolic acids during wheat beer brewing is mainly related to the enzymatic decarboxylation during fermentation and to a much lesser extent to the thermal decarboxylation during kilning, mashing, wort boiling, and flash pasteurisation. The contents of the desired vinyl aromatics and undesired, toxicologically relevant styrene mainly depend on the yeast strain applied for fermentation. Clearly lower styrene concentrations can be expected for Pof^- or $Pof^{+/-}$ *S. cerevisiae* strains, which, however, is accompanied by also lower concentrations of desired 4VP and 2M4VP. In fact, this will lead to wheat beers with a less pronounced overall aroma expected by the consumers.

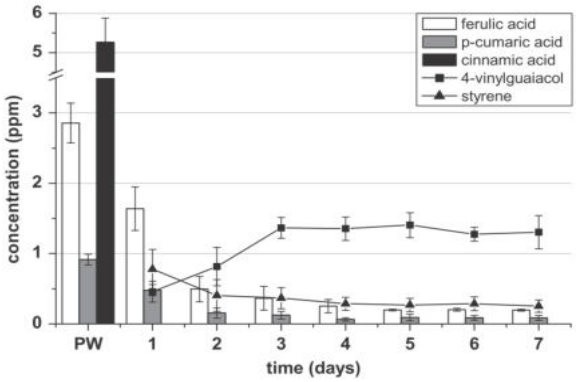
1.5.2 The Impact of Fermentation

Fermentation is the last step of the brewing process and as highlighted above (Section 1.5.1), it is during this processing step when nearly all of the phenolic acids are converted into the corresponding vinyl aromatics. A study by Wackerbauer et al. [50] proved that this decarboxylation is related to the presence of yeasts in the fermentation process. However, they also showed that this decarboxylation ability was only found for top-fermenting yeasts [50]. A further study by Daly et al. [20] revealed that top-fermenting yeasts form a diverse group with yeast strains expressing the decarboxylation activity with different intensities. They can have either no (Pof^-), a reduced ($Pof^{+/-}$), or a very strong (Pof^+) Pof-activity. Unfortunately, the decarboxylation activity of the yeast strains similarly affects the undesired and desired precursors. Thus, high undesired styrene and desired vinyl aromatic concentrations are obtained in the presence of yeast strains with a strong Pof-activity, while low contents of all vinyl aromatics are the result, of yeast strains with a reduced or no Pof-activity. Hence, the use of distinct yeast strains would allow the reduction of the styrene content, but this would always be accompanied by a severe reduction of the desired vinyl aromatics, and therefore, would lead to wheat beers lacking the characteristic aroma expected by consumers. Interested in the impact of further fermentation parameters, Schwarz et al. [22, 24] investigated the influence of the fermentation temperature, the pitching rate, and the fermentation technique (open vs. closed) on the formation of the vinyl aromatics. The analysis of the free phenolic acid and vinyl

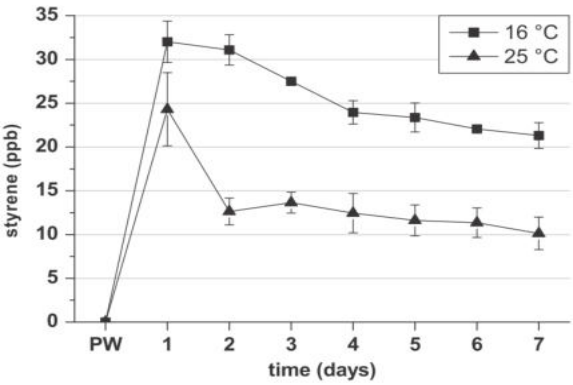
aromatic concentrations in 1 day intervals during the fermentation of wort at 16 ℃ (Figure 1.6a) and 25 ℃ (Figure 1.6b) over a time range of 7 days revealed that maximum styrene contents were reached already after after 1 day. In contrast, maximum 2M4VP concentrations were reached after 3 days at 25 ℃ and after 6 days at 16 ℃. Therefore, the decarboxylation of the precursors was much faster at 25 ℃ compared to 16 ℃, which can be addressed to a higher activity of the decarboxylases at higher temperatures. Interestingly, in case of free ferulic acid, both temperatures led to the same final concentrations of the 2M4VP, whereas in case of free cinnamic acid, styrene contents at 16 ℃ were higher at all days compared to 25 ℃ (Figure 1.6c). Furthermore, styrene showed a significant decrease after reaching its maximum, which was not found for 2M4VP. This decrease was found for both fermentation temperatures, however with a steeper slope at 25 ℃.



(a) Fermentation of a pitching wort (PW) spiked with cinnamic acid (5 μg/L) at 16 ℃.



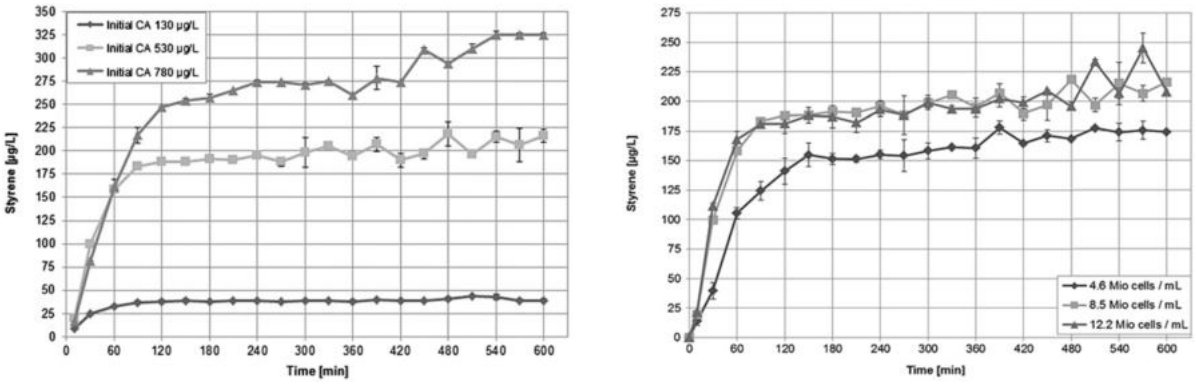
(b) Fermentation of a pitching wort (PW) spiked with cinnamic acid (5 μg/L) at 25 ℃.



(c) Styrene formation at 16 ℃ and 25 ℃ without the addition of additional cinnamic acid.

Figure 1.6: Influence of the fermentation temperature on the formation of the vinyl aromatics. Figures **1.6a** and **1.6b** depict the course of the degradation of the phenolic acids and the formation of the vinyl aromatics during fermentation of the same wort, spiked with 5 μg/L of cinnamic acid, at different fermentation temperatures of 16 ℃ and 25 ℃ over a time period of 7 days. Figure **1.6c** shows the concentration of styrene of the same wort during a fermentation without the addition of cinnamic acid. (PW pitching wort; Figures reprinted from Schwarz et al. [24] with permission from Elsevier.)

Monitoring the styrene concentration during the fermentation of a wort with 500 μg/L of free cinnamic acid and a pitching rate of $7.8 * 10^6$ cells/mL by Schwarz et al. [22] revealed that the formation of styrene followed a very short and steep increase at the beginning and after reaching the maximum, the concentration remained constant. This formation pattern showed that the formation of styrene immediately starts after pitching and no adaptation of the yeast to the conditions in media is necessary. In a further experiment, Schwarz et al. varied the free cinnamic acid concentration in wort from 130 via 530 to 780 μg/L and fermented the corresponding wort with the same pitching rate of $8.5 * 10^6$ cells/mL (Figure 1.7a). With increasing concentrations of free cinnamic acid, the steep initial phase was extended, and after reaching the maximum, the curves flattened and the concentrations remained quite constant. Within this experiment, a positive correlation between the free cinnamic acid content in the pitching wort and the styrene content in green beer was observed. In an additional experiment, the free cinnamic acid content was kept constant (520 μg/L), while the pitching rate was varied between 4.6, 8.5, and $12.2 * 10^6$ cells/mL (Figure 1.7b). Under these conditions, the slope proved to be steeper the higher the pitching rate was. Independent of the free cinnamic acid content and the pitching rate, most of free cinnamic acid was converted within 120 min. And although the free cinnamic acid content and the pitching rate showed these effects in the initial phase of the styrene formation, only the available amount of free cinnamic acid in the pitching wort did have an impact on the overall styrene concentration after full fermentation. Again, these findings highlight the importance of using malts with low amounts of cinnamic acid.

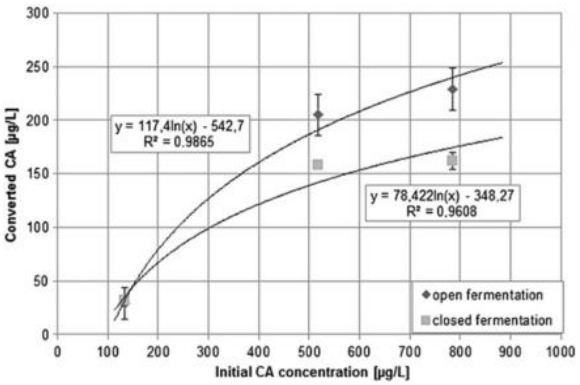


(a) Constant pitching rate with varying cinnamic acid concentrations.

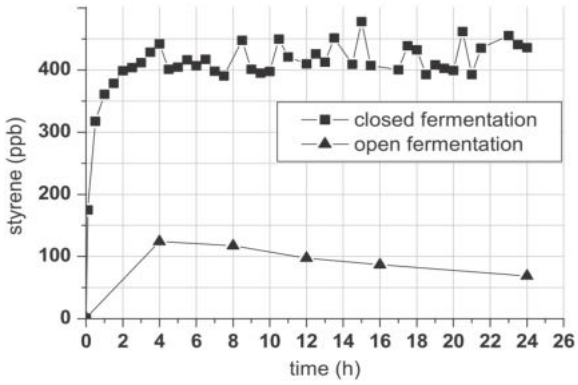
(b) Constant cinnamic acid concentration with varying pitching rates.

Figure 1.7: Influence of the pitching rate on the formation of styrene. Figure **1.7a** depicts the styrene formation during fermentation of three wort with different free cinnamic acid contents of 130, 530, and 780 μg/L at an equal pitching rate of $8.5 * 10^6$ cells/mL. Figure **1.7b** shows the formation of styrene during fermentation of a wort with 520 μg of free cinnamic acid/L at three different pitching rates of 4.6, 8.5, and $12.2 * 10^6$ cells/mL. (Figures reprinted from Schwarz et al. [22] with permission from Wiley.)

Comparing open and closed fermentations, Schwarz et al. [22, 24] showed that within the first 60 min, styrene contents were higher in an open fermentation compared to a closed fermentation (Figure 1.8a). This observation was ascribed to a higher enzymatic activity in an open fermentation, as the result of constant steering leads to a constant aeration and a homogeneous distribution of yeast in contrast to a lower enzymatic activity in a closed fermentation, as a consequence of the emerging pressure. This was in accordance with the results of Jones et al. [77], who showed that an increase of the CO_2 pressure was accompanied by a downregulation of the yeast metabolism and its cell division during fermentation. Further, Vanbeneden et al. [78] proved that an increasing pressure and lower temperatures disfavour the formation of 2M4VP. However, with an increasing fermentation time, these effects were shown to lose their impact. After 24 h, the styrene contents in a closed fermentation were 5 times higher than in an open fermentation (Figure 1.8b). As explanation, an increased evaporation of styrene, accelerated by the stripping off with CO_2, was quoted by Schwarz et al. [24].



(a) Short-term comparison of styrene formation during closed and open fermentation.



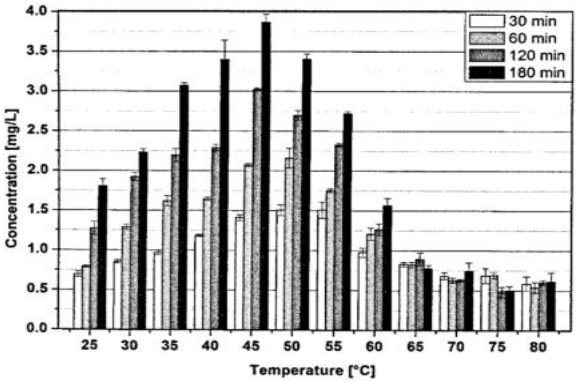
(b) Long-term comparison of styrene formation during closed and open fermentation.

Figure 1.8: Comparison of styrene formation during closed and open fermentation. Figure **1.8a** represents the logarithmic correlations between the initial cinnamic acid concentration in pitching wort and converted cinnamic acid after 60 min of a closed and an open fermentation management (pitching rate of $8.5 * 10^6$ cells/mL). Figure **1.8b** compares the styrene formation of a closed and open fermentation on a long-term scale of 24 hours. (Figures reprinted from Schwarz et al. [22, 24] with permission from Elsevier and Wiley.)

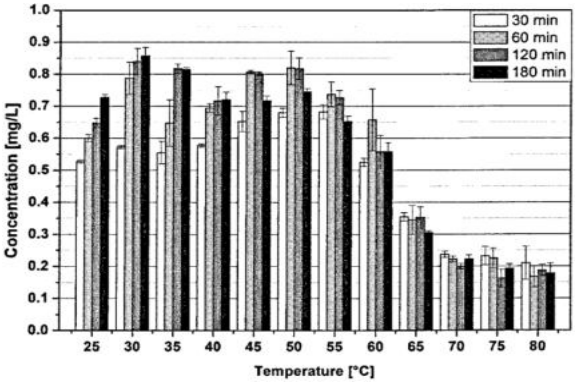
1.5.3 The Impact of Mashing

Mashing has a significant impact on the amounts of cinnamic, *p*-coumaric, and ferulic acid, which are later on accessible to yeast either in a free or soluble ester-bound form, as during this second step in wheat beer production phenolic acids are transferred from malt into wort. Additionally, enzymes directly or indirectly involved in the release of these precursors during malting are still active and lead to a further degradation of the arabinoxylan as well as to their direct release. Therefore, Schwarz et al. [23] investigated the impact of the mashing-in

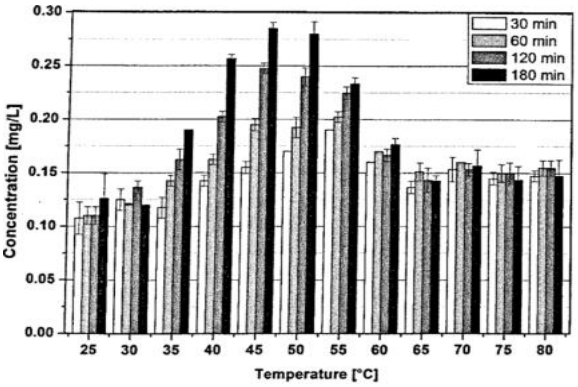
temperature, pH value, and time on the release of the desired and undesired phenolic acids. For their study, they used a mixture of Pilsner and wheat malt in a ratio of 1 + 1, which was coarse milled and then mashed-in by isothermal conditions.



(a) Ferulic acid release vs. mashing-in temperature.



(b) *p*-Coumaric acid release vs. mashing-in temperature.

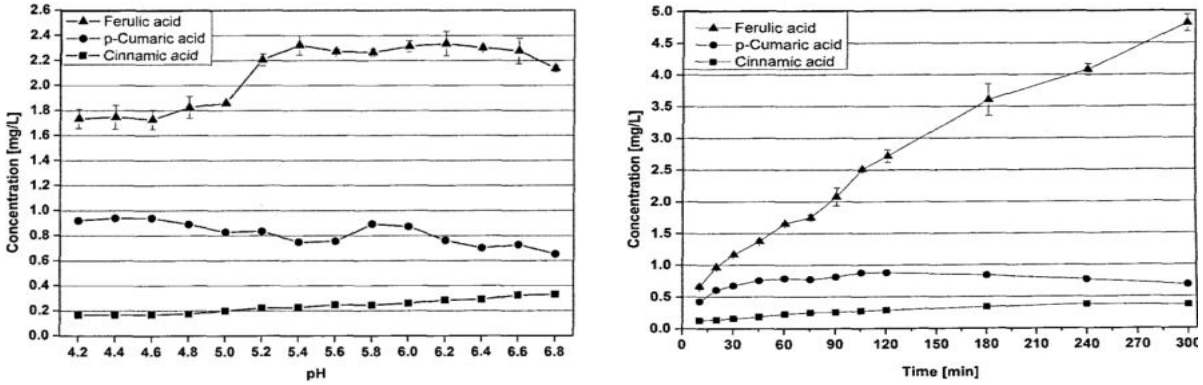


(c) Cinnamic acid release vs. mashing-in temperature.

Figure 1.9: Phenolic acid release as a function of the mashing-in temperature. The dependency of the release of free cinnamic (**1.9a**), *p*-coumaric (**1.9b**), and ferulic acid (**1.9c**) on the mashing-in temperature is shown between 25 ℃ and 80 ℃ in steps of 5 degrees. (Figures reprinted from Schwarz et al. [23] with permission from Taylor and Francis.)

In order to examine the effect of the mashing-in temperature, mashing experiments were conducted between 25 ℃ and 80 ℃ in steps of 5 degrees. The highest amounts of free ferulic acid were found at 45 ℃ and 50 ℃ (Figure 1.9a). Also for cinnamic acid, these temperatures proved to be best for an effective release (Figure 1.9c). In contrast, for *p*-coumaric acid no temperature optimum could be identified. Within the range from 25 ℃ to 60 ℃, a high release of *p*-coumaric acid was found (Figure 1.9b). For temperatures >60 ℃, contents remained constant for ferulic acid, while for cinnamic and *p*-coumaric acid the concentrations decreased. Additionally, for samples taken from these mashes after 30, 60, 120, and 180 min, it could be shown that the contents of cinnamic and ferulic acid increased with an increasing mashing-in

time, while the *p*-coumaric acid contents were unaffected. However, at temperatures >60 °C, time also had no effect on the concentrations of cinnamic and ferulic acid, indicating that enzymes involved in the release of phenolic acids where degenerated at these temperatures. Thus, the released amounts of phenolic acids should correspond to the soluble fraction in malt. Interestingly, the temperature dependency of the release of cinnamic and ferulic acid matches very well the temperature dependency of the feruloyl esterase acitivity [79].



(a) Phenolic acid release vs. mashing-in pH value. **(b)** Phenolic acid release vs. mashing-in time.

Figure 1.10: Phenolic acid release in dependency of the mashing-in pH value and time. Figure **1.10a** depicts the free phenolic acid concentrations in dependency of the pH value in the range between 4.2 and 6.8 in steps of 0.2 pH units after 60 min. Figure **1.10b** shows the free phenolic acid concentrations in dependency of time between 0 and 300 min in steps of 15 min at a pH value of 5.4. (Figures reprinted from Schwarz et al. [23] with permission from Taylor and Francis.)

The influence of the mashing-in pH value was studied in the range of 4.2 to 6.8 in steps of 0.2 units for samples taken after 60 min. In case of ferulic acid, contents were similar in mashes with a pH in the range of 4.2 to 5.0. At pH values of 5.0 to 5.2, a strong increase was found, with again comparable concentrations between 5.2 and 6.8. In contrast, a small decrease was found for *p*-coumaric acid, while cinnamic acid increased slightly with an increasing pH. However, the effect of the pH value seemed to be strongest for ferulic acid and less relevant for cinnamic and *p*-coumaric acid (Figure 1.10a).

To get a better understanding of the mashing-in time, the malt mixture was mashed-in at 45 °C and a pH value of 5.4, and samples were taken over a time period of 300 min, every 15 min. For ferulic acid, the impact of time was highly distinct, as a strong increase with time was found, expected to continue after 300 min. In contrast, the concentrations of cinnamic and *p*-coumaric acid increased only at a very small rate with increasing time and reached a maximum after 240 and 120 min, respectively. In case of cinnamic acid, contents remained at the same level with an increasing mashing-in time, while the contents of *p*-coumaric acid decreased slowly after reaching the maximum (Figure 1.10b).

1.5.4 The Impact of Malting

Enzymes are the workhorses in beer brewing, needed for the degradation of starch and proteins to make sugars and amino acids available for yeasts. All the relevant enzymes for the brewing process are endogenous in grain of barley and wheat. However, at the beginning of the brewing process, first, their expression in grain has to be triggered. This is achieved via germination, which is part of the malting process. Also enzymes directly or indirectly involved in the release of phenolic acids are endogenous to the grain of barley and wheat and are expressed during malting. This makes the malting process a very promising candidate for styrene mitigation in wheat beer. To get a first idea of the potential of this parameter, Langos et al. [26] studied the impact of the malting parameters germination temperature, germination time, and steeping degree on the release of free phenolic acids.

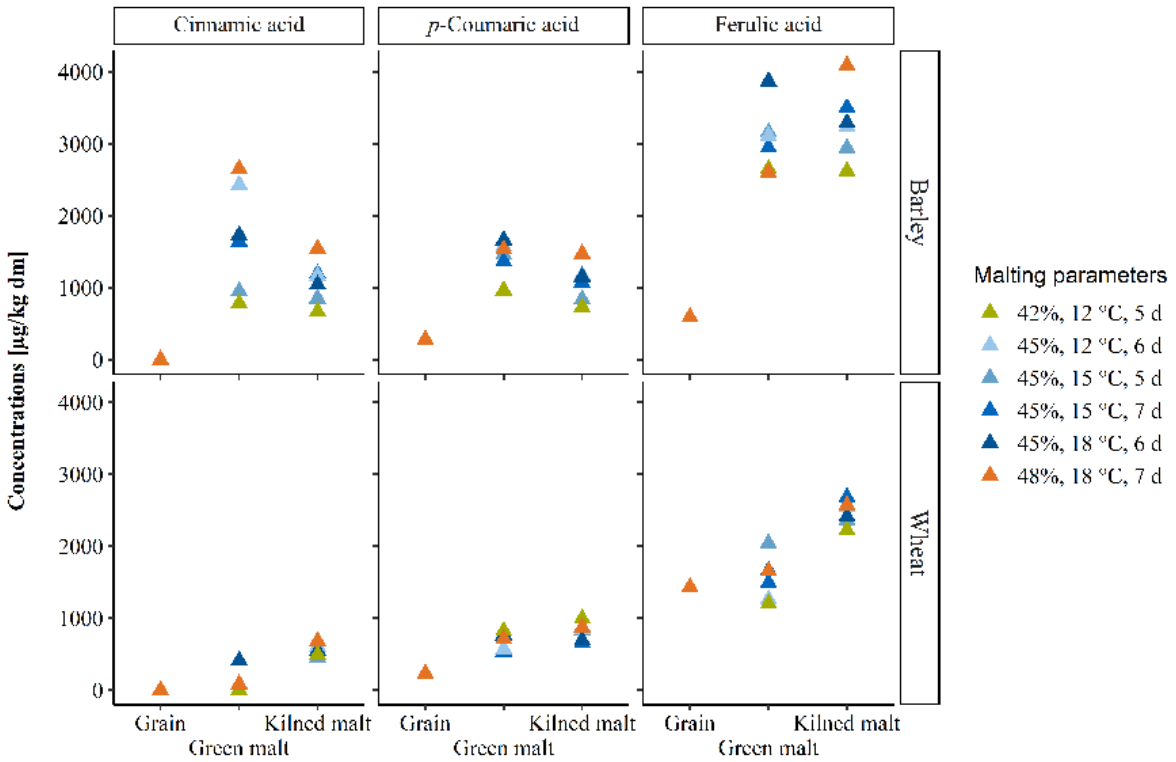


Figure 1.11: Impact of malting on the free phenolic acid contents. Minimum and maximum values of the malting parameters steeping degree (42, 48%), germination temperature (12, 18 °C), and germination time (5, 7 days) were applied during malting of one barley and one wheat variety to investigate the ability of the malting process to reduce the content of undesired cinnamic acid in the brewing process. ("Highly modified" malts are highlighted in orange and "undermodified" malts in green; raw data from Langos et al. [26].)

Within their study, one barley ("Hermann") and one wheat variety ("Marthe") were malted separately at a high and low value for each of the three parameters and samples were taken from

malts before and after kilning. A comparison of the malts produced with a germination time of 5 and 7 days revealed a slight increase of the free phenolic acid contents with and increase of time (Figure 1.11; 45%, 15 °C, 5 d vs. 45%, 15 °C, 7 d). In case of the wheat variety, the contents of cinnamic and ferulic acid in kilned malts increased from 0.45 to 0.48 μg/kg dm and from 2.35 to 2.68 μg/kg dm, respectively, whereas the content for *p*-coumaric acid decreased slightly from 0.83 to 0.65 μg/kg dm. For the barley variety, the impact was slightly stronger, with an increase of the cinnamic acid content from 0.85 to 1.19 μg/kg dm, of *p*-coumaric acid from 0.84 to 1.07 μg/kg dm, and of ferulic acid from 2.94 to 3.50 μg/kg dm.
The impact of the germination temperature was studied at 12 and 18 °C. Although the differences in the free phenolic acids contents were very small, there seemed to be a tendency of a decrease in the amounts of the precursors with an increase of the germination temperature (Figure 1.11; 45%, 12 °C, 6 d vs. 45%, 18 °C, 6 d). For the wheat variety, the contents in kiln-dried malts decreased for cinnamic acid from 0.61 to 0.54 μg/kg dm, and for *p*-coumaric and ferulic acid from 0.84 to 0.69 μg/kg dm and from 2.54 to 2.41 μg/kg dm, respectively. Among the barley varieties, the decrease was even less pronounced with 1.17 to 1.04 μg/kg dm for cinnamic acid and 1.17 to 1.15 μg/kg dm for *p*-coumaric acid, while a slight increase from 3.24 to 3.29 μg/kg dm was observed for ferulic acid.
Within the third experiment, studying the impact of the steeping degree, also minimum and maximum values of the parameters germination temperature and time were applied creating "highly modified" (Figure 1.11; 48%, 18 °C, 7 d; in orange) and "undermodified" malts (Figure 1.11; 42%, 12 °C, 5 d; in green). Kiln-dried malts of the wheat variety showed slightly higher free phenolic acid contents within the highly modified malts compared to the undermodified malts. Cinnamic acid and ferulic acid contents increased from 0.49 to 0.68 μg/kg dm and from 2.22 to 2.57 μg/kg dm, while *p*-coumaric acid contents decreased from 0.99 to 0.87 μg/kg dm. In comparison, the impact was again more pronounced for the barley variety, where the free cinnamic, *p*-coumaric, and ferulic acid contents increased from 0.67 to 1.57 μg/kg dm, from 0.73 to 1.47 μg/kg dm, and from 2.62 to 3.35 μg/kg dm. Therefore, the use of the undermodified wheat malt would allow the reduction of the free cinnamic acid content by about 30% compared to the highly modified wheat malt, whereas in case of the barley variety, even a reduction of >50% was achievable. However, the results also showed that the different precursors are equally affected by these parameters, whereupon a reduction of cinnamic acid would also lead to a reduction of the desired phenolic acids.
In a following study [29], the highly modified and undermodified malts were used for micro-brewing trials (Figure 1.12). Interestingly, for barley, the concentration differences in the malts were not reflected by the amounts found in cast wort, where the phenolic acid contents were much more comparable (211 vs. 208 μg/L for cinnamic acid, 562 vs. 297 μg/L for *p*-coumaric acid, 1,330 vs. 1,020 μg/L for ferulic acid). Fermentation of these wort with Pof-active yeasts led to green beers with similar amounts of styrene (18.6 vs. 18.0 μg/L); however, much lower contents of the desired vinyl aromatics in case of the undermodified malt (235 vs. 159 μg/L

for 4VP, 1,100 vs. 695 μg/L for 2M4VP) were found. Although only minor differences were analyzed for the wheat malts, the differences in the phenolic acid contents in the corresponding cast wort were huge (281 vs. 153 μg/L for cinnamic acid, 1,030 vs. 345 μg/L for *p*-coumaric acid, 3,200 vs. 2,240 μg/L for ferulic acid). In fact, this also led to great differences in the contents of the vinyl aromatics in the corresponding green beers, where now not only the desired vinyl aromatics were affected (724 vs. 202 μg/L for 4VP, 2,640 vs. 2,280 μg/L for 2M4VP), but also styrene showed significantly lower contents (25.4 vs 17.5 μg/L). In summary, the use of undermodified wheat malt led to a reduction of styrene in the corresponding beer of around 30%, whereas the use of undermodified barley malt had no effect. For both undermodified barley and wheat malts the desired vinyl aromatics were significantly lowered. However, the remaining concentrations of 4VP and 2M4VP were still high enough to express the typical wheat beer aroma, because in each case, the contents of the desired vinyl aromatics were still above their respective threshold as well as over the 600 μg/L limit for 2M4VP, which was found to be necessary in a wheat beer to elicit the typical aroma expected by the consumers [8].

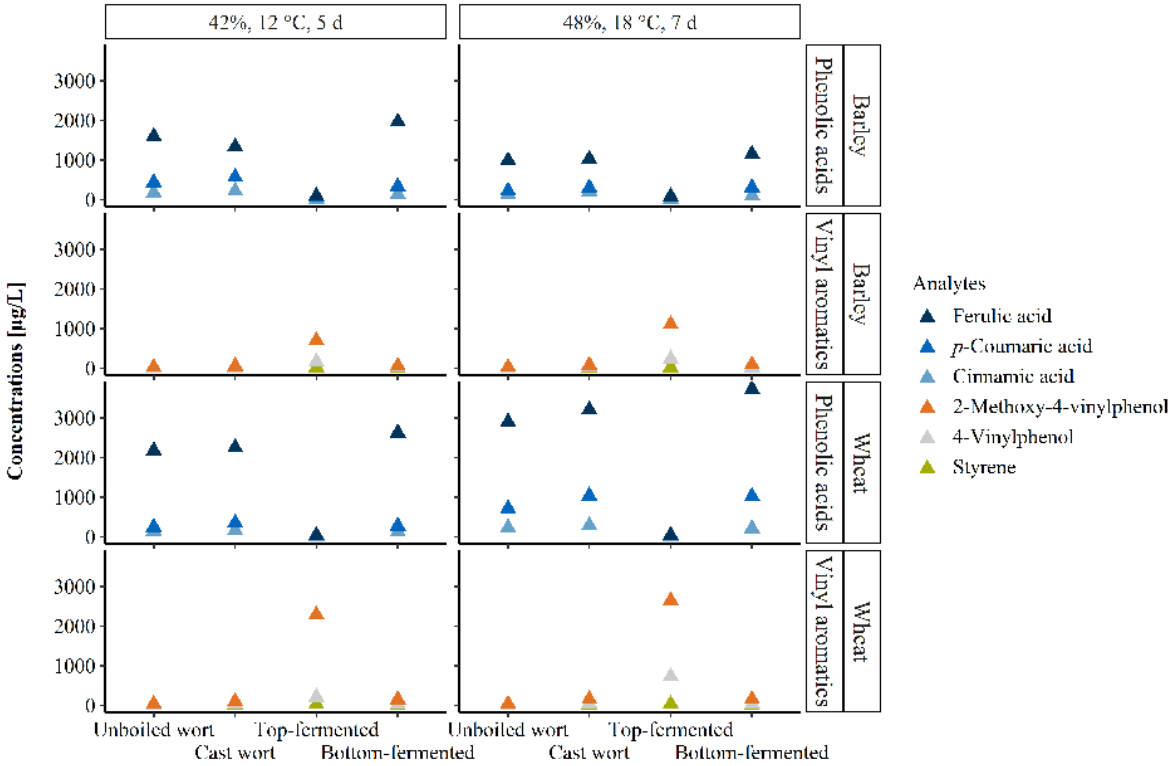


Figure 1.12: Micro-brewing trials of highly modified and undermodified malts. Phenolic acid and vinyl aromatic patterns of micro-brewing trials from barley and wheat malts produced with either intensive (48%, 18 °C, 7 d) or moderate (42%, 12 °C, 5 d) malting parameters. Increasing germination temperature and time were found to intensify cytolytic modifications of malt, while higher steeping degrees were found to intensify proteolytic modifications [80].

2 Motivation and Objectives

Due to the carcinogenic toxicity of styrene and its occurrence as a natural component in wheat beer, there is a huge interest of the brewing industry for mitigation strategies. Previous studies, investigating the suitability of the various processing steps of wheat beer brewing for styrene reduction, revealed the malting process to be a very interesting candidate. Therefore, this thesis aimed at a detailed understanding of the impact of malting of barley and wheat on the formation and release of the precursors of the desired and undesired vinyl aromatics during the production of wheat beer.

To evaluate whether the implementation of the knowledge of the aforementioned studies led to an improvement of the food safety of wheat beer, first, free phenolic acid and desired and undesired vinyl aromatic concentrations were analysed in 20 commercially available wheat beers. The data were further used to calculate possible correlations between the precursors and the decarboxylation products, as well as between the desired vinyl aromatics and styrene. Additionally, dark, nonalcoholic, and reduced-alcohol wheat beers as well as bottom-fermented lager beers were included in the sample set to study the influence of roasting, dealcoholisation, and the yeast type on the styrene content and on the concentration pattern of the analytes of interest in wheat beers.

Aiming at a general understanding of the role of malting and mashing on the precursor contents, malts and wort of four barley and three wheat varieties were prepared applying typical conditions used in wheat beer production. As previous mitigation studies on styrene in wheat beer found no correlations between the free phenolic acid contents in any of the processing steps and the final concentrations of the toxicologically relevant styrene and the desired aroma-active vinyl aromatics in wheat beer, caused by the feruloyl esterase being still active after kiln-drying [75] and the yeast's own feruloyl esterase activity [76], additionally to the free precursor contents, the total and soluble contents were determined in these grain, malt, and wort samples. From the data obtained, further research questions were answered with respect to the total, soluble, and free cinnamic, *p*-coumaric, and ferulic acid contents: how do differences and changes in the contents depend on the grain type and the variety; how do the amounts change upon malting and mashing; what are the transfer rates from malt into wort; how do malting and mashing affect the ratio of insoluble ester-bound, soluble ester-bound, and free precursor contents; and how do malting and mashing influence the ratio of cinnamic to *p*-coumaric and ferulic acid? In case of cinnamic acid, insoluble and soluble ester-bound contents have yet not been reported in literature. Therefore, with the new data at hand, this knowledge gap should be closed.

That phenolic acids exist in an insoluble ester-bound, soluble ester-bound, and free form, is the consequence of degradation processes occurring during germination, which is part of the malting process. Thereby, various enzymes degrade the cell wall polysaccharide arabinoxylan, to which the precursors are preliminary bound, into fragments of different sizes and therefore, with different solubilities, or directly release the phenolic acids. With the objective of getting a deeper insight into the enzyme activities, directly or indirectly involved in the release of phenolic acids, grain and malt samples should be analysed for their α- and β-amalyse, β-glucanase, feruloyl esterase, and protease activities, as well as their total and water-extractable arabinoxylan and β-glucan contents. In particular, these results were used to answer the following research questions: how does a possible partial degradation of the cell walls impact the release of free and soluble ester-bound phenolic acids; how does the solubilisation of arabinoxylan affect the release of free and soluble ester-bound phenolic acids; is there a correlation between the feruloyl esterase activity and the release of free phenolic acids; and does the protease activity influence enzymes directly or indirectly involved in the release of phenolic acids?

In malting experiments, selected parameters, including the steeping temperature, the conditions of germination temperature and aeration rate, and the temperatures applied during kiln-drying, were tested for their suitability for styrene mitigation. Therefore, two barley varieties and one wheat variety, relevant to the brewing industry, were chosen for the respective malting experiments. Since the transfer of the changes in the malts to the wort was of further interest, these malt samples were additionally mashed-in. Finally, the malt and wort samples were analysed for the most interesting analytical compounds: the total, soluble, and free phenolic acid contents, the amounts of total and water-extractable arabinoxylan, and the feruloyl esterase activity. When evaluating the suitability of the malting parameters for styrene mitigation, the maintenance of sufficient amounts of the desired precursors should always have been included in the judgement, due to the similar biochemical formation pathway of styrene and the desired vinyl aromatics.

Up to now, barley and wheat varieties have always been studied separately. Concerning possible interactions of the phenolic acid contents and enzymatic activities between these two grain types mixed during wheat beer production, wort was prepared from different percentages of wheat malt in the grain bill. Additionally, based on the yeast's own feruloyl esterase and the missing correlation between the free phenolic acid contents in process intermediates to the vinyl aromatic contents in wheat beer, wort was further fermented in presence of two different Pof-active top-fermenting yeast strains, addressing the question to what extent soluble ester-bound phenolic acids in wort contribute to the final vinyl aromatic contents in wheat beer. Finally, ultra high-performance liquid chromatography-time-of-flight-mass spectrometry (UHPLC-ToF-MS) with the technique of Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS) was applied to extracts of malts containing soluble ester-bound phenolic acids and MS-fragmentation rules of polysaccharides were used to get an idea about the structure of these soluble precursor fragments.

3 Publications

3.1 Formation of Desired Smoky Key Odorants in Wheat Beer: A Comparison with the Undesired Toxicologically Relevant Styrene

3.1.1 Summary

The following study aimed at investigating the concentration range of styrene in commercially available wheat beers and at finding out whether the implementation of the knowledge of former mitigation studies by breweries has led to an improvement of the food safety of wheat beer. Additionally, also the contents of the desired vinyl aromatics and of the corresponding precursors were subject to this market survey. A special focus was further set on the comparison of the analyte pattern in pale wheat beers with those in dark, reduced-alcohol, and nonalcoholic wheat beers as well as bottom-fermented lager beers, to clarify the impact of roasting, dealcoholisation, and the yeast type on the analyte pattern.

The styrene content in regular pale and dark wheat beers was found to be between 9.8 and 31.6 μg/L. Market surveys prior to the former mitigation studies revealed a similar concentration range of styrene in wheat beers, indicating that the implementation of the gained knowledge by the breweries either did not happen or had no significant impact on the reduction of styrene. Indeed, this highlighted the importance of further studies on the complex topic of styrene mitigation.
Within the sample set, a positive correlation was found between the aroma-active analytes and styrene, proving that increasing amounts of the desired vinyl aromatics are accompanied with increasing amounts of styrene. Between the vinyl aromatics and their precursors, a negative

correlation was found, highlighting that increasing concentrations of the volatile analytes are accompanied with a decrease of the content of the phenolic acids. The causality of this observation is linked to the presence of top-fermenting yeasts, which are able to convert phenolic acids via decarboxylation into the corresponding vinyl aromatics.
Low concentrations of styrene and the desired vinyl aromatics in reduced-alcohol wheat beers were found to be the consequence of a lower original gravity and not due to an evaporation of the volatile vinyl aromatics during thermal dealcoholisation. The reduction of the vinyl aromatics is therefore caused by the reduced amount of malt used for the production of reduced-alcohol wheat beers, which not only reduces the amount of ethanol via a lower input of sugars but also leads to a lower input of phenolic acids. In case of the nonalcoholic wheat beers, a stopped fermentation could be ascribed as the applied dealcoholisation method. Beside the reduced concentration of the desired vinyl aromatics, these wheat beers showed an unexpected high styrene concentration, which can be explained by the fact that during a normal fermentation maximum styrene contents are reached already after 2 h of fermentation, whereas maximum contents of 4VP and 2M4VP are reached after 3 to 6 days.
Dark wheat beers were found to have significantly lower vinyl aromatic contents compared to regular pale wheat beers. As dark wheat beers are brewed with an original gravity similar to regular pale wheat beers, the reduction of the desired vinyl aromatics as well as of styrene can be ascribed to a reduced input of the corresponding precursors by the use of dark malts. In fact, studies proved that high kilning temperatures, used for the production of dark malts, led to a thermal decarboxylation of phenolic acids, and therefore, to a reduction of these precursors in dark malts.
Vinyl aromatic and phenolic acid patterns of Pof^+, $Pof^{+/-}$, and Pof^- yeast strains perfectly demonstrated the impact of the decarboxylation ability of these yeast types. For Pof^+ yeasts, a nearly complete decarboxylation of the phenolic acids into their corresponding vinyl aromatics was found, while $Pof^{+/-}$ and Pof^- yeasts showed only a slight conversion of the precursors with slightly higher contents of the vinyl aromatics in case of the $Pof^{+/-}$ yeasts.

The individual contributions of V. Kalb and the co-authors' contributions are specified below:

V. Kalb developed the design of the study and conducted the laboratory work. Mass spectrometric determination of phenolic acids via HPLC-MS/MS was performed by V. Kalb with the help of S. Kaviani-Nejad, Leibniz-Institute for Food System Biology at the Technical University of Munich. Mass spectrometry of vinyl aromatics via GCxGC-ToF-MS was executed by V. Kalb with the help of I. Otte, Leibniz-Institute for Food System Biology at the Technical University of Munich. V. Kalb statistically analysed and interpreted the data, discussed the results, and created the figures and tables. He developed the story of the paper, wrote the first draft of the manuscript, and revised the paper. Prof. Dr. M. Granvogl, Institute of Food Chemistry, University of Hohenheim, was responsible for the conceptualization, project administration, supervision of the research project, and writing reviews and editing.

3.1.2 Publication

Formation of Desired Smoky Key Odorants in Wheat Beer: A Comparison with the Undesired Toxicologically Relevant Styrene

V. Kalb and M. Granvogl

Permanent weblink: http://dx.doi.org/10.1021/bk-2019-1321.ch008

Chapter 8

Formation of Desired Smoky Key Odorants in Wheat Beer: A Comparison with the Undesired Toxicologically Relevant Styrene

Valerian Kalb[1] and Michael Granvogl[*,2,3,4]

[1]Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Lise-Meitner-Straße 34, Freising 85354, Germany

[2]Lehrstuhl für Lebensmittelchemie, Technische Universität München, Department für Chemie, Lise-Meitner-Straße 34, D-85354 Freising, Germany

[3]Lehrstuhl für Analytische Lebensmittelchemie, Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Maximus-von-Imhof-Forum 2, D-85354 Freising, Germany

[4]Institut für Lebensmittelchemie, Fachgebiet für Lebensmittelchemie und Analytische Chemie (170a), Universität Hohenheim, Fakultät Naturwissenschaften, Garbenstrasse 28, D-70599 Stuttgart, Germany

[*]E-mail: Michael.Granvogl@ch.tum.de.

Wheat beer is a special beer type that is traditionally brewed in Bavaria (southern part of Germany) and Austria. By law, it must be brewed with a wheat malt content of at least 50%. Its characteristic aroma has been described as clovelike and slightly phenolic and is elicited by the presence of 4-vinylphenol and 2-methoxy-4-vinylphenol, two desired decarboxylation products from *p*-coumaric acid and ferulic acid (both cinnamic acid derivatives). Unfortunately, wheat beer also contains an undesired decarboxylation product, namely styrene (vinylbenzene), which is formed via the same pathway from cinnamic acid. Former studies of the brewing process have shown that these compounds are mainly formed by yeast during fermentation from the aforementioned precursors, traditionally referred to as phenolic acids. Despite this knowledge, the current market survey based on 20 commercially available wheat beer samples from different breweries revealed styrene concentrations ranging from 0.22 to 31.6 µg/L, which were similar to amounts reported in previous studies.

Introduction

Wheat beer is a specialty beer type that is very popular in Bavaria (Germany) and Austria and by law must be brewed with a wheat malt content of at least 50%. The aroma of wheat beer has been described as clovelike and slightly phenolic, evoked by 2-methoxy-4-vinylphenol (2M4VP) with a clovelike and smoky odor impression and 4-vinylphenol (4VP) with an almond-shell-like and phenolic smell (*1*). Wheat beer with relatively low concentrations of these compounds exhibits a less pronounced typical aroma. Furthermore, in addition to these desired volatile vinyl aromatics, the undesired compound styrene (S) is naturally present in typical wheat beers.

Evidence of styrene in beer goes back to the 1970s (*2*, *3*), but first attention to its toxicological relevance was not reported until 1996, when the World Health Organization published a tolerable daily intake for drinking water of 7.7 µg/kg body weight per day (*4*). Then, in 2002, styrene was classified by the International Agency of Research on Cancer as possibly carcinogenic to humans (class 2B) based on the results of studies on mice (*5*) and rats (*6*). On the basis of these facts, even if the reported average styrene concentrations in wheat beers will not lead to an excess of the tolerable daily intake, which is enforced for drinking water, mitigation strategies should be applied.

The aforementioned vinyl aromatics have a common biochemical formation pathway, namely the enzymatic decarboxylation of the precursors cinnamic acid (C), *p*-coumaric acid (*p*C), and ferulic acid (F). The conversion of these, traditionally referred to as phenolic acids, occurs mainly in presence of Pof^+ active top-fermenting yeast strains during fermentation in wheat beer production (*7*, *8*). In crops, phenolic acids have an important functional role, as they link arabinoxylan chains to a three-dimensional network. This building block, together with β-glucans, structures the endosperm cell wall of the grains. Thus, barley and wheat are natural sources for these precursor acids.

Several studies by Langos et al. (*1*, *9*–*11*), Langos (*12*), and Schwarz et al. (*8*, *13*–*16*) examined the influence of different process steps of brewing on the release of styrene and the desired vinyl aromatics. It was shown that thermal process steps, like mashing, wort boiling, and pasteurization, have little to no contribution to the overall styrene concentration caused by thermal decarboxylation. Comparative studies on open versus closed fermentation revealed reduced styrene concentrations (−25%) using the former. Styrene is thereby stripped off with the CO_2 that is formed, whereas in a closed fermentation, vaporized styrene condenses again without any loss. Nevertheless, open fermentation plays only a minor role in brewing these days.

Known yeast strains exhibit no difference in their selectivity against the phenolic acid precursors. Thus, high concentrations of styrene correlated with high concentrations of 4VP and 2M4VP and vice versa. This is in accordance with the results of Daly et al. (*17*), who classified yeasts into three phenotypes, Pof^+, $Pof^{+/-}$, and Pof^-, according to their ability to decarboxylate phenolic acids. Because styrene mitigation strategies within both the thermal-processing steps and fermentation were always accompanied by the loss of the desired vinyl phenolic odorants, further studies focused on the impact of malting conditions on the release of the precursors (*11*, *18*). Thereby, the use of "undermodified" malts (malting parameters: steeping degree, 45%; germination temperature, 12 °C; germination time, 5 days) clearly reduces the content of undesired cinnamic acid compared with the contents of the desired phenolic acids, making the malting process a promising tool to lower the styrene content in wheat beer.

Table 1 summarizes past market studies on the concentration of styrene, as well as 4VP and 2M4VP, in commercially available wheat beers. This chapter investigated, in a renewed study on 20 commercially available wheat beers, whether the implementation of the gained knowledge by

breweries has led to an improvement of the food safety of wheat beer. Therefore, the aims of the current study were to analyze the precursors *p*-coumaric acid, ferulic acid, and cinnamic acid (using liquid chromatography-tandem mass spectrometry [LC-MS/MS]) as well as the corresponding decarboxylation products 4VP, 2M4VP, and styrene (using comprehensive gas chromatography time-of-flight mass spectrometry [GCxGC-ToF-MS]) based on stable isotope dilution assays. With these data in hand, correlations between the precursors and the decarboxylation products, as well as between the desired vinyl aromatics and styrene, should be calculated.

Table 1. Published Data on Styrene Concentrations in Commercial Wheat Beers

				Concentration [µg/L] of		
Literature	*Fermentation*	*Pof. act.*[a]	*S*	*4VP*	*2M4VP*	*n*
Langos et al. 2016 (*10*)	Top	+	15–33	620–1020	630–2020	6
	Top, r.-alc.[b]	+	25	355	795	1
Daly et al. 1997 (*17*)	Top	+	25–31	—	—	4
	Top, r.-alc.[b]	+	9, 25	—	—	2
	Top	–	<0.04	—	—	3
	Bottom	–	<0.04	—	—	7
	Bottom, r.-alc.[b]	–	<0.04	—	—	9
Wackerbauer et al. 1982 (*19*)	Top	+	—	1250	2517	1
	Bottom	–	—	10	98	1
	Special beers	n.a.[c]	—	555–5204	187–251	3
Wackerbauer et al. 1982 (*20*)	Top	+	—	440–2700	520–4300	21
	Top	n.a.[c]	—	20	189	1

[a] Pof activity. [b] Reduced-alcohol. [c] Not available.

Experimental Section

Materials

Twenty different wheat beers from 16 Bavarian (Germany) breweries were purchased from a local store in October 2018. All wheat beers were bottled in 500 mL amber glass bottles and were stored at room temperature. Eighteen of the 20 wheat beers were unfiltered, pale wheat beers with an alcohol content of between 4.9 and 5.8 vol.-%. Additionally, the sample set included two dark wheat beers (both 5.3 vol.-%), two reduced-alcohol (2.8 and 3.3 vol.-%), and two nonalcoholic (<0.5 vol.-%) wheat beers. For comparison with the top-fermented wheat beers, two bottom-fermented lager beers (“Helles”) were also analyzed.

The following stable isotopically labeled internal standards were commercially obtained: [$^{13}C_3$]-ferulic acid (99 atom % ^{13}C), [$^{13}C_3$]-*p*-coumaric acid (99 atom % ^{13}C), [$^{2}H_7$]-cinnamic acid (98 atom % ^{2}H), and [$^{2}H_8$]-styrene (98 atom % ^{2}H) were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany); [$^{2}H_3$]-2-methoxy-4-vinylphenol (99.5 atom % ^{2}H) was from Toronto Research Chemicals (North York, ON, Canada). [$^{2}H_5$]-4-Vinylphenol was synthesized according to Jezussek (*21*).

All solvents were of HPLC gradient grade; acetonitrile was from Baker (Sowińskiego, Poland), formic acid from Merck, and ultrapure water from an in-house source.

Quantitation of Free Phenolic Acids in Wheat Beer by High-Performance Liquid Chromatography-Tandem Mass Spectrometry Based on Stable Isotope Dilution Analysis

Naturally cloudy wheat beer samples were degassed and clarified by filtration (paper filter, 5 H/N, 240 mm, 85 g/m^2; Sartorius, Göttingen, Germany) and by an additional ultrasonification step (10 min). Reduced-alcohol and regular wheat beers were used without dilution, lager beers and nonalcoholic wheat beers were diluted 1:5 (v:v) with water prior to further sample preparation steps. Afterward, the stable isotopically labeled internal standards [$^{13}C_3$]-ferulic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{2}H_7$]-cinnamic acid (amounts determined in preliminary experiments) were added and the samples were equilibrated by a multitube vortexer (VWR, Darmstadt) for 15 min. Subsequently, the samples were membrane-filtrated (Minisart RC, hydrophilic, 15 mm; Sartorius), and the phenolic acids were quantitated via high-performance liquid chromatography-tandem mass spectrometry.

High-performance liquid chromatography-tandem mass spectrometry analysis was performed using an UltiMate 3000 HPLC system (Thermo Fisher Scientific; Dionex Softran, Germering, Germany) coupled to a triple quadrupole mass spectrometer (TSQ Vantage, ThermoFisher Scientific, Bremen, Germany). A Kinetex C18 column (100 × 2.1 mm, 2.6 μm, 10.0 nm) (Phenomenex, Aschaffenburg, Germany) was applied for the separation of the precursor acids using the following conditions: solvent A, aqueous formic acid (FA) (0.1%, v/v), solvent B, FA (0.1%, v/v) in acetonitrile; gradient: 0–2 min, 10% B; 2–15 min, from 10 to 90% B; 15–18 min, 90% B; 18–19 min, from 90 to 10% B; 19–30 min, 10% B; flow rate, 0.2 mL/min; injection volume, 10–20 μL; column temperature, 24 °C. The ion source was operated in positive atmospheric pressure chemical ionization mode (APCI$^+$) using the following parameters: discharge current, 4.0 μA; vaporizer temperature, 250 °C; sheath gas pressure, 30 arbitrary units; auxiliary gas pressure, 10 arbitrary units; declustering voltage, −10 V; capillary temperature, 300 °C. Selected reaction monitoring (SRM) was used to analyze the transitions from precursor to product ions using experimentally optimized collision energies.

Quantitation of Volatile Vinyl Aromatics in Wheat Beer by Headspace-Solid Phase Microextraction in Combination with Comprehensive Gas Chromatography-Time-of-Flight-Mass Spectrometry Based on Stable Isotope Dilution Analysis

Wheat beer samples were prepared as mentioned above for the analysis of the precursor acids. Sodium chloride (2 g), tap water (4 mL), and wheat beer (1 mL) were mixed in a headspace vial (20 mL) equipped with a magnetic stir bar. Lager beers (5 mL) were analyzed without a dilution with water. Then, the stable isotopically labeled internal standards [$^{2}H_3$]-2-methoxy-4-vinylphenol,

[$^2H_5$]-4-vinylphenol, and [$^2H_8$]-styrene (amounts determined in preliminary experiments) were added, the headspace vial was immediately sealed (silicon, PTFE septum screw caps), and the sample was stirred for 1 h on a magnetic stirrer at room temperature to adjust the sample headspace equilibrium.

Headspace-solid phase microextraction was performed using a Gerstel MultiPurpose Sampler equipped with a DVB/CAR/PDMS-coated fiber (50/30 µm 2 cm; Supelco; Bellefonte, PA, USA). Prior to sample analysis, a blank run was performed each day to bake out the fiber. The SPME parameters were set as follows: incubation temperature, 50 °C; incubation time, 3.0 minutes; extraction temperature, 50 °C; extraction time, 30 minutes. The adsorbed analytes were thermally desorbed in the injection port of the gas chromatography-time-of-flight-mass spectrometry system at 250 °C for 32 min.

The headspace-solid phase microextraction analysis was performed using a Leco Pegasus 4D gas chromatography-time-of-flight-mass spectrometry instrument (St. Joseph, MI) comprising an Agilent model 7890A GC, a dual-stage quad-jet thermal modulator, and a secondary oven coupled to the mass spectrometer providing unit mass resolution (Waldbronn, Germany). An Agilent Multimode Inlet operated in splitless mode for thermal desorption of the analytes in combination with a Gerstel MultiPurpose Sampler autosampler (Mülheim a.d. Ruhr, Germany) were used. Separation was achieved by a DB-FFAP fused silica column (30 m × 0.25 mm i.d., 0.25 µm film thickness, equipped with a deactivated precolumn (2 m × 0.53 mm i.d.; both J&W, Agilent) in the first dimension and a VF-5-MS column (2 m × 0.15 mm i.d., 0.3 µm film thickness; Varian, Darmstadt) in the second dimension. The column mode was set to constant flow (1.90 mL/min). The first oven temperature program was: 35 °C for 5 min, raised by 6 °C/min to 230 °C, and held for 5 min. The second oven was started at 60 °C for 5 min, then raised at 6 °C/min to 250 °C, and finally kept for 5 min. The modulator offset was set to + 40 °C. Mass spectra were acquired via electron ionization (70 eV) over a mass range *m/z* 35–300 at a rate of 100 spectra/s. Data were processed using GC Image (Lincoln, NE).

Statistical Analysis

Calculation of mean values and standard deviations were performed by Microsoft Office Excel 2007 (Microsoft Corporation, Seattle, WA) and Pearson correlation coefficients by R 3.4.3 (RStudio, Boston, MA).

Results and Discussion

Vinyl Aromatic Contents of 20 Commercial Wheat Beers

Styrene, 4-vinylphenol, and 2-methoxy-4-vinylphenol concentrations were measured in 26 beer samples: 18 pale wheat beers, two dark wheat beers, two reduced-alcohol wheat beers, two nonalcoholic wheat beers, and two bottom-fermented lager beers used as "negative" controls. The results, summarized in Table 2, showed a concentration range for styrene in regular pale and dark wheat beers from 9.8 to 31.6 µg/L, for 4VP from 434 to 1350 µg/L, and for 2M4VP from 620 to 2490 µg/L, respectively. Thereby, wheat beers 3 and 5 differed strongly from the remaining regular wheat beer samples due to their significantly lower vinyl aromatic concentrations, which were 0.22 and 0.70 µg/L for styrene, 65.4 and 55.7 µg/L for 4VP, and 140 and 109 µg/L for 2M4VP, respectively.

Table 2. Volatile Vinyl Aromatics and Free Phenolic Acids of 18 Pale Wheat Beers (3, 7–9, 11, 14–26), Two Dark Wheat Beers (D; 5, 10), Two Reduced-Alcohol Wheat Beers (AR; 6, 12), Two Nonalcoholic Wheat Beers (AF; 4, 13), and Two Bottom-Fermented Beers (BF; 1, 2) from 17 Different Breweries (Ascending Order of Styrene Concentration)

		Concentration[a] [µg/l] of					
Sample		*Styrene*	*4-Vinylphenol*	*2-Methoxy-4-vinylphenol*	*Cinnamic acid*	*p-Coumaric acid*	*Ferulic acid*
1	BF	<LoQ[b]	31.1	79.5	95.5	423	1200
2	BF	<LoQ	29.4	81.4	97.0	500	1390
3		0.22	65.4	140	79.6	1150	2040
4	AF	0.57	40.1	77.7	81.4	1010	1550
5	D	0.70	55.7	109	101	1180	2150
6	AR	1.78	31.9	60.5	65.4	744	1340
7		9.75	829	2490	<LoD[c]	11.1	353
8		11.4	1010	1650	<LoD	4.87	23.6
9		12.1	1220	1450	<LoD	10.1	35.8
10	D	12.1	434	620	<LoD	7.11	39.1
11		12.4	816	1660	<LoD	22.9	81.2
12	AR	13.3	648	900	<LoD	6.66	28.3
13	AF	14.2	506	372	<LoD	356	660
14		15.0	969	2070	5.97	76.7	198
15		16.0	560	1090	<LoD	4.45	17.7
16		16.1	1020	1390	<LoD	3.70	15.5
17		18.3	896	1420	2.69	9.50	58.7
18		19.8	860	1160	<LoD	4.65	76.0
19		20.1	1050	1280	<LoD	3.59	11.3
20		20.4	803	1790	<LoD	4.53	22.9

Table 2. (Continued). Volatile Vinyl Aromatics and Free Phenolic Acids of 18 Pale Wheat Beers (3, 7–9, 11, 14–26), Two Dark Wheat Beers (D; 5, 10), Two Reduced-Alcohol Wheat Beers (AR; 6, 12), Two Nonalcoholic Wheat Beers (AF; 4, 13), and Two Bottom-Fermented Beers (BF; 1, 2) from 17 Different Breweries (Ascending Order of Styrene Concentration)

	Concentration[a] [µg/l] of					
Sample	*Styrene*	*4-Vinylphenol*	*2-Methoxy-4-vinylphenol*	*Cinnamic acid*	*p-Coumaric acid*	*Ferulic acid*
21	21.7	628	1260	3.37	2.69	20.3
22	22.6	750	1370	<LoQ[d]	12.5	38.0
23	24.7	1290	1880	2.91	24.0	69.6
24	27.9	1080	2120	<LoD	4.64	26.3
25	28.8	1120	1830	<LoD	4.25	22.2
26	31.6	1350	1900	<LoD[c]	4.86	19.9

[a] All results are mean values of triplicates. Average relative standard deviations are 2.5% for styrene, 1.6% for 4-vinylphenol, 1.9% for 2-methoxy-4-vinylphenol, 6.7% for cinnamic acid, 3.8% for *p*-coumaric acid, and 4.0% for ferulic acid. [b] LoQ = 0.10 µg/L. [c] LoD = 0.75 µg/L. [d] LoQ = 2.51 µg/L.

According to Wackerbauer et al. (*20*), wheat beers with <600 µg/L of 2M4VP elicit an atypical to neutral wheat beer aroma (e.g., wheat beers 3 and 5). Within the current sample set, 18 of the 20 wheat beers and one reduced-alcohol wheat beer exhibited a typical wheat beer aroma, as was sensorially assessed during sample preparation (data not shown). This correlates with concentrations >620 µg/L found for 2M4VP in these samples. For "negative" controls, two pale lager beers were analyzed, as the flavor of this beer type does not typically have any of the odor attributes of wheat beer. This is linked to the low amounts of vinyl aromatics, with approximately 30 µg/L of 4VP, 80 µg/L of 2M4VP, and styrene concentrations <LOQ (0.10 µg/L) found here in beers 1 and 2.

Correlation of the Styrene Content with the Amount of Desired Vinyl Aromatics as well as the Amount of Free Cinnamic Acid in Wheat Beer

Pearson correlation coefficients were calculated to determine the relationship between styrene, the desired vinyl aromatics, and the free phenolic acids in the analyzed beer samples (Table 3). Within the decarboxylation products, correlation coefficients >0.75 were found, indicating that an increase of styrene is linked to an increase of 4VP and 2M4VP. By comparison, a positive correlation was also found within the set of free phenolic acids, with correlation coefficients >0.88. A comparison of both groups with each other revealed a negative correlation, with correlation coefficients <−0.75; thus, a decrease of free phenolic acids is related to an increase of styrene, 4VP, and 2M4VP. To corroborate these results, the null hypothesis was tested, and a high significance was confirmed by p-values $\leq 1.0 \times 10^{-5}$.

Table 3. Pearson Correlation Coefficients between Vinyl Aromatics and Phenolic Acids in the Analyzed Set of Wheat and Lager Beers

	S	*4VP*	*2M4VP*	*C*	*pC*	*F*
S	1					
4VP	0.84[a]	1				
2M4VP	0.75[a]	0.88[a]	1			
C	−0.80[a]	−0.84[a]	−0.78[a]	1		
*p*C	−0.75[a]	−0.80[a]	−0.77[a]	0.88[a]	1	
F	−0.81[a]	−0.84[a]	−0.77[a]	0.94[a]	0.98[a]	1

[a] p-Value $\leq 1.0 \times 10^{-5}$.

The causality of these correlations is linked to the presence of yeast. Wackerbauer et al. (*22*) proved that yeasts are able to convert *p*-coumaric acid and ferulic acid into their corresponding vinyl aromatics. The decarboxylation ability of yeasts to convert cinnamic acid into styrene was later shown by Goddey et al. (*7*). Thus, the negative correlation can be explained by the conversion of free phenolic acids, extracted from the malts into the pitching wort, into the corresponding vinyl aromatics by yeasts during fermentation. The positive correlation within the vinyl aromatics highlights an additional fact, namely the lack of selectivity of yeasts against the phenolic acids. This lack in selectivity was also found by Daly et al. (*17*), who analyzed the release of 2M4VP and styrene from different yeast strains and POF types. Consequently, changing the yeast strain is not an appropriate option to reduce the styrene concentration, as the characteristic odor impression of wheat beer will also be lost. Comparison of the results of wheat beers 3 and 9 (cf. Table 2) highlights

that lower concentrations of styrene are accompanied by lower amounts of 4VP and 2M4VP and vice versa (Figure 1). Interestingly, both wheat beers (3 and 9) were from the same brewery and differed only in the yeast strain used for fermentation.

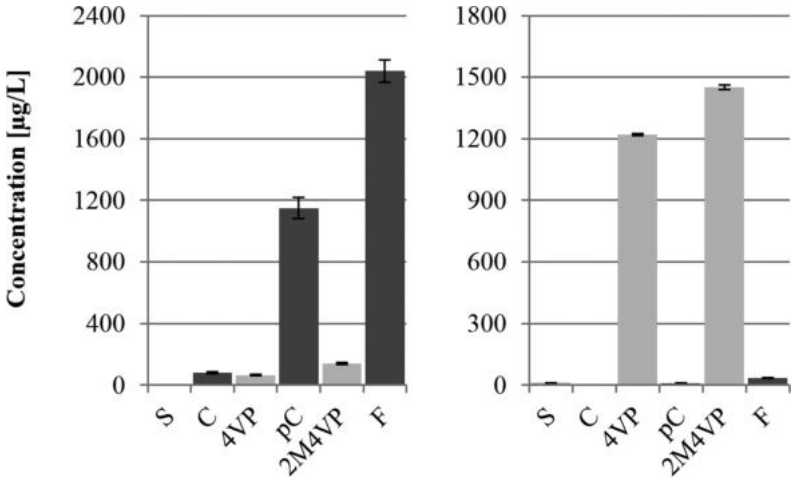


Figure 1. Wheat beer brewed with a yeast strain with strongly reduced Pof$^{+/-}$activity (left). Wheat beer brewed with the standard Bavarian wheat beer yeast strain (right).

To the best of our knowledge, wheat beer breweries in Bavaria usually use the same top-fermenting yeast strain for fermentation, except one brewery that uses a different strain (wheat beers 3 to 6 in Table 2). Therefore, different ratios between styrene, 4VP, and 2M4VP within the remaining set of pale wheat beer samples in Table 2 are influenced by other factors, for example, barley and wheat varieties used (*23*, *24*), malting conditions applied (*12*), malt ratio used (*8*, *25*), or mashing conditions applied (*13*, *26*). All of them may affect the input of free phenolic acids into the brewing process as well as the overall fermentation management (*14*, *15*, *27*). This explains why the calculated Pearson correlation coefficients of a dataset including only pale wheat beers showed no significant correlations.

Effect of Dealcoholization Process on the Styrene Concentration in Wheat Beers

For the dealcoholization process of beer, either fermentation is stopped when the desired alcohol content is reached (stopped fermentation) or the alcohol is removed after the complete fermentation. For the latter, different methods are applied (e.g., thermal dealcoholization, reducing the alcohol content by evaporation, or reverse osmosis, removing the alcohol via a semipermeable membrane). Schwarz et al. (*15*) proved a nearly complete conversion of cinnamic acid into styrene after 2 h after starting the fermentation by addition of Pof$^{+/-}$ active yeasts. Therefore, it is expected that a stopped fermentation has no effect on the styrene concentration but does reduce the content of the desired vinyl aromatics as they reach their maximum of release after 3 to 6 days, depending on the fermentation temperature (*14*). In case of thermal dealcoholization, a clearly reduced amount of all vinyl aromatics is expected, with the lowest content for styrene due to its volatility and its low initial concentration. Regarding the precursors, a reverse effect for the stopped fermentation will occur, leading to high remaining concentrations. For the case of methods that remove the alcohol after fermentation, the amount of free phenolic acids will remain low.

Table 4 compares the vinyl aromatic and free phenolic acid composition of a regular, a reduced-alcohol, and a nonalcoholic wheat beer from the same brewery using the standard yeast strain. The regular wheat beer, with an original gravity of 12.8% and an alcohol content of 5.4%, showed a normal wheat beer pattern of precursors and vinyl aromatics. Regarding the reduced-alcohol wheat beer, the low amount of free phenolic acids indicated that the fermentation was completed. However, the alcohol content of 3.3% was adjusted in this case by a lower original gravity of 7.8% instead of removing the alcohol from the regular wheat beer. With the lower original gravity, less cinnamic acid was introduced into the wort, which explains the lowered styrene concentration compared with the regular wheat beer. The nonalcoholic wheat beer, with an original gravity of 6.5%, had a similar styrene concentration as the reduced-alcohol wheat beer and lower concentrations of 4VP and 2M4VP. Further, it showed high amounts of free *p*-coumaric and ferulic acid, but no cinnamic acid, which is exactly the pattern assumed for a reduced-alcohol wheat beer brewed by stopped fermentation, as a complete conversion of cinnamic acid into styrene, but just a partial conversion of the precursors into 4VP and 2M4VP, based on the reduced fermentation time, was found. The lower concentration of 2M4VP compared with 4VP may lead to the assumption that the reactivity of the decarboxylase is higher for *p*-coumaric acid compared with ferulic acid.

Table 4. Styrene Content in a Reduced-Alcohol and a Nonalcoholic Wheat Beer Compared with a Regular Wheat Beer from the Same Brewery

	Concentration[a] [µg/L] of					
Type	*S*	*4VP*	*2M4VP*	*C*	*pC*	*F*
Wheat beer	24.7	1290	1880	2.91	24.0	69.6
Red.-alcohol	13.3	648	900	<LoD[b]	6.66	28.3
Nonalcoholic	14.2	506	372	<LoD	356	660

[a] All results are mean values of triplicates. [b] LoD = 0.75 µg/L.

Impact of Roasting Conditions on the Styrene Concentration in Wheat Beers

A further comparison of the data revealed higher concentrations of styrene and desired vinyl aromatics in a pale wheat beer compared with a dark wheat beer from the same brewery (Table 5). In both samples, the low free phenolic acid contents point to the fact that a complete fermentation took place. Thus, the lower styrene and desired vinyl aromatic contents in the dark wheat beer must be related to a lower input of the corresponding free phenolic acids by the malt into the wort. Samaras et al. (*28*) determined the relationship between the amounts of free *p*-coumaric acid and ferulic acid to the roasting degree of barley malts. With increasing kilning temperature, a massive decrease of free phenolic acids was found, which can be explained by the high temperatures used during kilning (220– 229 °C). To prove whether this finding was due to a thermal decarboxylation, the authors exemplarily analyzed the 2M4VP concentration in the respective malts. Thereby, malts kilned with a temperature <140 °C did not contain any 2M4VP, whereas malts kilned above 220 °C exhibited concentrations of 2M4VP of 267 to 439 µg/kg malt per dry mass. As both wheat beers, pale and dark, have almost the same original gravity of approximately 12.8%, a lower input of free phenolic acids as in reduced-alcohol wheat beers is not the explanation for the lower vinyl aromatic contents. As such, the lower amounts of styrene and desired vinyl aromatics in dark wheat beer can be explained by a loss of the precursor during kilning of dark malts, as shown by Samara and co-workers (*28*). Regarding the vinyl aromatics generated at high kilning temperatures, these results suggested that

they are lost within the thermal processing steps due to the fact that they were not transferred into the beer, which is evident from the lower amounts found in dark wheat beer.

Table 5. Styrene Content in One Pale and One Dark Wheat Beer from the Same Brewery

	Concentration[a] [µg/L] of					
Type	*S*	*4VP*	*2M4VP*	*C*	*pC*	*F*
Pale wheat beer	20.4	803	1790	< LoD[b]	4.53	22.9
Dark wheat beer	12.1	434	620	< LoD	7.11	39.1

[a] All results are mean values of triplicates. [b] LoD = 0.75 µg/L.

Influence of the Yeast Type on the Styrene Content in Wheat Beers

The ability of yeast strains to convert phenolic acids into their corresponding volatile vinyl aromatics is linked to the presence of the POF1 gene (*7*). POF stands for phenolic off-flavor because originally these vinyl aromatics were undesired in beers and mostly occurred when wild yeast strains were unknowingly involved in the fermentation process. However, this conversion ability of yeasts to decarboxylate the precursors is now desired, leading to the characteristic aroma of wheat beer.

Daly et al. (*17*) found that bottom-fermenting yeasts lack the ability to decarboxylate phenolic acids, consequently called Pof$^-$. In this regard, top-fermenting yeasts were found to be an inhomogeneous group. Those with a high Pof activity are classified as Pof$^+$ and those with a very low conversion rate are Pof$^{+/-}$; also, top-fermenting yeasts with no Pof activity have been found. Analysis of three beers fermented with yeast strains of different Pof activities highlighted the influence of this characteristic on the pattern of vinyl aromatics and free phenolic acids (Table 6). In this study, Pof$^-$ was represented by a bottom-fermented lager beer. According to the absent decarboxylation activity, the free phenolic acids were still high, and nearly no vinyl aromatics were formed after the brewing process. The small amounts can be explained by thermal decarboxylation during the thermal processing steps, like mashing, wort boiling, and pasteurization (*10*). Pof$^+$ was represented by a pale wheat beer. Due to the high Pof activity, the decarboxylation of the free phenolic acids took place during fermentation, resulting in high amounts of the desired and undesired vinyl aromatics. Pof$^{+/-}$ was represented also by a pale wheat beer; however, this brewery used a traditional in-house yeast strain, which shows only a very low Pof activity, leading to the low vinyl aromatic concentrations and, correspondingly, to the very high remaining precursor amounts. The same yeast strain was also used for fermentation of wheat beers 3 to 6 (cf. Table 2), which explains their different vinyl aromatic and free phenolic acid pattern.

Table 6. Effect of the Yeast Type on the Styrene Content in Beer

	Concentration[a] [µg/L] of					
Yeast type	*S*	*4VP*	*2M4VP*	*C*	*pC*	*F*
Pof$^-$	<LoQ[b]	31.1	79.5	95.5	423	1200
Pof$^{+/-}$	0.22	65.4	140	79.6	1150	2040
Pof$^+$	12.1	1220	1450	<LoD[c]	10.1	35.8

[a] All results are mean values of triplicates. [b] LoQ = 0.10 µg/L. [c] LoD = 0.75 µg/L.

A comparison of the vinyl aromatic concentrations found in this study with the published data summarized in Table 1 indicated that there was no mitigation of styrene in most of the commercially available wheat beers over the past years. Therefore, further cooperation and investigations on the complex topic of styrene reduction in wheat beer brewing should be done.

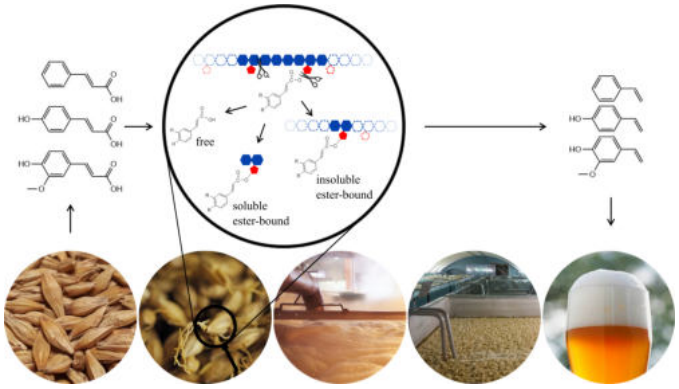
References

1. Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. *J. Agric. Food Chem.* **2013**, *47*, 11303–11311.
2. Renner, R.; Bartels, H.; Tressl, R. Enrichment and quantitative determination of beer aroma substances by gas extraction. *Monatsschr. Brau.* **1976**, *29*, 478–480.
3. Tressl, R.; Friese, L.; Fendesack, F.; Koeppler, H. Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. *J. Agric. Food Chem.* **1978**, *6*, 1422–1426.
4. *Guidelines for Drinking-Water Quality: Health Criteria and Other Supporting Information*, 2nd ed.; World Health Organization: Geneva, 1996.
5. Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Bevan, C.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J. Appl. Toxicol.* **2001**, *21*, 185–198.
6. Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicol. Sci.* **1998**, *46*, 266–281.
7. Goddey, A. R.; Tubb, R. S. Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *J. Gen. Microbiol.* **1982**, *128*, 2615–2610.
8. Schwarz, K.; Methner, F.-J. Styrene concentrations during wheat beer production. *Brew. Sci.* **2011**, *64*, 156–158.
9. Langos, D.; Granvogl, M.; Meitinger, M.; Schieberle, P. Development of stable isotope dilution assays for the quantitation of free phenolic acids in wheat and barley and malts produced thereof. *Eur. Food Res. Technol.* **2015**, *5*, 637–645.
10. Langos, D.; Granvogl, M. Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. *J. Agric. Food Chem.* **2016**, *11*, 2325–2332.
11. Langos, D.; Gastl, M.; Granvogl, M. Reduction of toxicologically relevant styrene in wheat beer using specially produced wheat and barley malts. *Eur. Food Res. Technol.* **2017**, *193*, 558–568.
12. Langos, D. *Optimierung technologischer Parameter zur Minimierung der Bildung von 4-Vinylbenzol (Styrol) beim Brauprozess unter Erhalt des typischen Aromas von Weizenbier (in German)*. Ph.D. thesis, Technical University of Munich, Freising, Germany, 2017.
13. Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Release of phenolic acids and amino acids during mashing dependent on temperature, pH, time and raw materials. *J. Am. Soc. Brew. Chem.* **2012**, *70*, 290–295.
14. Schwarz, K. J.; Stübner, R.; Methner, F.-J. Formation of styrene dependent on fermentation management during wheat beer production. *Food Chem.* **2012**, *4*, 2121–2125.

15. Schwarz, K. J.; Boitz, L. I.; Methner, F.-J. Enzymatic formation of styrene during wheat beer fermentation is dependent on pitching rate and cinnamic acid content. *J. Inst. Brew.* **2012**, *3*, 280–284.
16. Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Influence of mashing conditions on the release of precursors of phenolic wheat beer aroma. *Brauwelt* **2013**, *153*, 274–279.
17. Daly, B.; Collins, E.; Madigan, D.; Donnelly, D.; Coakley, M.; Ross, P. An investigation into styrene in beer. *Proc. Congr.—Eur. Brew. Conv.* **1997**, *26*, 623–630.
18. Langos, D.; Granvogl, M.; Gastl, M.; Schieberle, P. Influence of malt modifications on the concentrations of free phenolic acids in wheat and barley malts. *Brew. Sci.* **2015**, *68*, 93–101.
19. Wackerbauer, K. Phenolic aromatic substances in beer: Phenolic carboxylic acid and phenols in the raw material and beer preparation. *Brauwelt* **1982**, *15*, 618–620.
20. Wackerbauer, K.; Krämer, P. Bavarian wheat beer - an alternative. Production and composition (in German). *Brauwelt* **1982**, *18*, 758–762.
21. Jezussek, M. M. *Zur Aromabildung beim Kochen von Naturreis (Oryza sativa L.) sowie Blättern von Pandanus amaryllifolius Roxb (in German)*. Ph.D. thesis, Technical University of Munich, Garching, Germany, 2002.
22. Wackerbauer, K.; Kossa, T.; Tressl, R. Phenol formation by yeasts (in German). *Monatsschr. Brauwiss.* **1978**, *31*, 52–55.
23. Vanbeneden, N.; Gils, F.; Delvaux, F.; Delvaux, F. R. Variability in the release of free and bound hydroxycinnamic acids from diverse malted barley (*Hordeum vulgare* L.) cultivars during wort production. *J. Agric. Food Chem.* **2007**, *26*, 11002–11010.
24. Cui, Y.; Wang, A.; Zhang, Z.; Speers, R. A. Enhancing the levels of 4-vinylguaiacol and 4-vinylphenol in pilot-scale top-fermented wheat beers by response surface methodology. *J. Inst. Brew.* **2015**, *1*, 129–136.
25. Coghe, S.; Benoot, K.; Delvaux, F.; Vanderhaegen, B.; Delvaux, F. R. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **2004**, *3*, 602–608.
26. Narziß, L.; Miedaner, H.; Nitzsche, F. The formation of 4-vinylguaiacol during the manufacture of Bavarian wheat beer (in German). *Monatsschr. Brauwiss.* **1990**, *3*, 96–100.
27. Kieninger, H.; Narziß, L.; Hecht, S. Changes of quality determining compounds in the preparation of Bavarian wheat beer (in German). *Monatsschr. Brauwiss.* **1984**, *1*, 9–18.
28. Samaras, T. S.; Camburn, P. A.; Chandra, S. X.; Gordon, M. H.; Ames, J. M. Antioxidant properties of kilned and roasted malts. *J. Agric. Food Chem.* **2005**, *20*, 8068–8074.

105

3.2 Studies on the Impact of Malting and Mashing on the Free, Soluble Ester-Bound, and Insoluble Ester-Bound Forms of Desired and Undesired Phenolic Acids Aiming at Styrene Mitigation during Wheat Beer Brewing



3.2.1 Summary

Addressing the drawback of former mitigation studies on styrene in wheat beer, analysing solely the free precursor contents and in addition the fact that no correlation between these contents in any of the process intermediates and the final vinyl aromatic contents in wheat beer has been found up to now, this study focused on a better understanding of the coherence between the free, soluble ester-bound, and insoluble ester-bound forms of phenolic acids during malting and mashing of barley and wheat varieties.

As a part of this study, high-throughput methods, based on the technique of stable isotope dilution assays, were developed for the determination of the total, soluble, and free phenolic acid contents in grain, malt, and wort. Besides the design of the sample preparation, this included the development of a new HPLC-MS/MS method as well as a validation of the method for the quantitation of free phenolic acids in grain and malt, on which basis all other methods were established.
Since the total and soluble cinnamic acid contents have been unknown up to now, this study was the first that determined the maximum available cinnamic acid contents in grain, malt, and wort. With total contents between 0.86–1.27 μg/kg dm in malt and soluble contents between 1.26–1.76 μg/kg dm in wort, concentration differences by factors of up to 2,000 in malts and up to 100 in wort were found between the maximum contents of cinnamic acid and the total contents of *p*-coumaric and ferulic acid in these process intermediates. Comparing the barley and wheat varieties, the barley varieties always showed slightly higher cinnamic acid contents, independently of the phenolic acid form and the processing step. However, as these differences were very low, a reduction of cinnamic acid, either by the use of different ratios of barley to wheat or by the use of different varieties, was found to be very limited within this study.

In case of cinnamic acid, all three forms were highly affected by malting and mashing, showing a constant increase from grain via malt to wort by factors between 1.3 and 12. Taking into account that the total cinnamic acid contents increased from grain to malt by factors between 2.9 and 3.9 and from malt to wort by factors between 1.3 and 2.2, malting had a stronger impact on the maximum cinnamic acid contents than mashing, highlighting the important role of malting in styrene mitigation. Different to cinnamic acid, total *p*-coumaric acid was only slightly affected by malting. And although malting and mashing had some decreasing and increasing effects on the free and soluble forms, these effects were negligible considering the huge concentration differences of total *p*-coumaric acid between unmalted barley and wheat. With these differences being in the range of roughly a factor of 10, soluble and free *p*-coumaric acid contents were directly related to the total contents, with always higher amounts in the barley varieties. Total ferulic acid contents in grain were comparable between the barley and wheat varieties, which was similar to cinnamic acid. Upon malting, a strong increase of the total ferulic acid contents occurred, while the soluble and free forms barely changed. During mashing, soluble ferulic acid contents decreased similarly in both grain types, with overall higher amounts in wheat wort.
In grain and malt, cinnamic acid occurred predominantly in a soluble form, whereas the desired precursors were mainly insoluble ester-bound. This had a direct effect on the transfer rates from malt into wort, which were found to be >100% for cinnamic acid, revealing that a complete transfer was accompanied by an additional biosynthesis. In contrast, transfer rates of the desired *p*-coumaric and ferulic acid were only <8%. Interestingly, in wort, cinnamic and *p*-coumaric acid contents were dominated by the free form, while ferulic acid was mostly still soluble ester-bound. Finally, a comparison of the soluble phenolic acid contents in wort revealed that the use of barley malts led to an introduction of cinnamic, *p*-coumaric, and ferulic acid into wort in a ratio of 2:14:84, and the use of wheat malt in a ratio of 1:2:97.

The individual contributions of V. Kalb and the co-authors' contributions are specified below:

V. Kalb developed the design of the study and conducted the laboratory work. Grain, malt, and wort samples were prepared by T. Seewald at the Chair of Brewing Science and Beverage Technology, Technische Universität Berlin. A. Köhler, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, assisted in sample preparation and S. Kaviani-Nejad, Leibniz-Institute for Food System Biology at the Technical University of Munich, was involved in the development of the HPLC-MS/MS method. V. Kalb processed and evaluated the data, discussed the results, and created the figures and tables. He developed the story of the paper, wrote the first draft of the manuscript, and revised the paper. T. Hofmann, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, coordinated the research project. Prof. Dr. M. Granvogl, Institute of Food Chemistry, University of Hohenheim, was responsible for the conceptualization, project administration, supervision of the research project, and writing reviews and editing.

3.2.2 Publication

Studies on the Impact of Malting and Mashing on the Free, Soluble Ester-Bound, and Insoluble Ester-Bound Forms of Desired and Undesired Phenolic Acids Aiming at Styrene Mitigation during Wheat Beer Brewing

V. Kalb, T. Seewald, T. Hofmann, and M. Granvogl

Permanent weblink: https://dx.doi.org/10.1021/acs.jafc.0c04835

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Studies on the Impact of Malting and Mashing on the Free, Soluble Ester-Bound, and Insoluble Ester-Bound Forms of Desired and Undesired Phenolic Acids Aiming at Styrene Mitigation during Wheat Beer Brewing

Valerian Kalb, Torsten Seewald, Thomas Hofmann, and Michael Granvogl*

Cite This: J. Agric. Food Chem. 2020, 68, 12421−12432 Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Mitigation studies on styrene in wheat beer found no correlation between the free phenolic acid contents in the processing steps and the final concentrations of the toxicologically relevant styrene and the desired aroma-active vinyl aromatics in beer, which can be explained by the presence of phenolic acid releasing enzymes that are still active after kiln-drying and by the yeast's own feruloyl esterase activity. The present study contributed to a better understanding of the coherence between the free, soluble ester-bound, and insoluble ester-bound forms of cinnamic, *p*-coumaric, and ferulic acid during malting and mashing of barley and wheat varieties. Concentration differences in malt by factors of up to 1700 were found between the total cinnamic acid contents (an undesired precursor of the toxicologically relevant styrene) and the total contents of *p*-coumaric and ferulic acid (both desired precursors of the aroma-active compounds 4-vinylphenol and 2-methoxy-4-vinylphenol). In grain and malt, cinnamic acid occurred predominantly in a soluble form, whereas the desired precursors were mainly insoluble ester-bound. This had a direct effect on the transfer rates from malt into wort, which were found to be >100% for cinnamic acid, revealing that a complete transfer was accompanied by an additional biosynthesis, but only <8% for the desired phenolic acids. Interestingly, in the wort, cinnamic and *p*-coumaric acid contents were dominated by the free form, while ferulic acid was mostly still soluble ester-bound. Overall, the use of barley malts led to an introduction of cinnamic, *p*-coumaric, and ferulic acid into the wort in a ratio of 2:14:84, and the use of wheat malt in a ratio of 1:2:97.

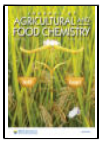
KEYWORDS: *styrene, vinyl aromatics, phenolic acids, wheat beer, malt, wort, stable isotope dilution assay*

INTRODUCTION

Wheat beer is a very popular beer type in Germany and Austria and has to be brewed with >50% of wheat malt. Different from other beer types, it elicits a characteristic clove-like and slightly phenolic aroma note, which is evoked by two key aroma compounds, namely, 4-vinylphenol and 2-methoxy-4-vinylphenol.[1] These two desired vinyl aromatics are released during the fermentation step in the brewing process in the presence of top-fermenting Pof$^+$-active yeasts[2,3] from the corresponding phenolic acid precursors (cinnamic acid derivatives), *p*-coumaric acid and ferulic acid. Besides these precursors, cinnamic acid, showing similar behavior as phenolic acids, naturally occurs in barley and wheat. In this case, decarboxylation by yeast leads to the formation of the undesired, toxicologically relevant styrene. Initial evidence of styrene in beer traces back to the 1970s.[4,5] However, its toxicological relevance was not considered until 1996 since the World Health Organization (WHO) established a tolerable daily intake (TDI) of 7.7 μg/kg body weight per day.[6] In 2002, styrene was labeled as a carcinogen (class 2B, possibly carcinogenic to humans) by the International Agency of Research on Cancer (IARC)[7] according to studies on rats[8] and mice.[9]

As a consequence, mitigation strategies had to be developed for foods containing styrene. Schwarz et al.[10−14] and Langos et al.[1,15−18] investigated the impact of the different processing steps of wheat beer, focusing on the release of undesired styrene, and also on the desired aroma-active vinyl aromatics. It is known that all of these compounds are mostly formed during the fermentation step, whereas thermal processing steps, such as mashing, wort boiling, and flash pasteurization, had a negligible effect on their concentrations.[14] The selected yeast strain plays a major role in whether the precursors are converted into the corresponding vinyl aromatics or not.[17,18] Daly et al.[2] classified yeast strains according to their ability to decarboxylate phenolic acids as Pof$^-$, Pof$^{+/-}$, or Pof$^+$. It was shown that bottom-fermenting yeasts lack, without any exception, the ability to convert phenolic acids, whereas top-fermenting yeasts can have either one of these Pof-activities. Additionally, it was proven that decarboxylases of Pof-active

Received: July 28, 2020
Revised: September 14, 2020
Accepted: September 18, 2020
Published: September 18, 2020



https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

yeast strains are very unselective and decarboxylate cinnamic, *p*-coumaric, and ferulic acid with similar conversion rates.[3] Thus, a reduction of styrene, using a yeast strain with a reduced or no Pof-activity, is always accompanied by a reduction of the desired aroma-active vinyl aromatics. However, too low concentrations of these key odorants lead to wheat beers without the typical aroma expected by the consumers. Therefore, the focus in further studies was on the reduction of styrene via the precursor cinnamic acid. An investigation on the impact of the mashing conditions on the release of the precursors revealed similar temperature optima for all phenolic acids between 45 and 50 °C.[12] With an increasing mashing-in time, also an increase of all free phenolic acids was found, although this effect was highly distinct for ferulic acid. Additionally, the pH value optimum for the release of ferulic acid was found to be between 5.4 and 6.6, whereas for *p*-coumaric acid only a slight decrease and for cinnamic acid a slight increase was found with an increasing pH value. Therefore, mashing is theoretically a first tool to reduce the styrene content, but its practical use is limited due to only slightly differentiated effects on the precursor concentrations. Further processing steps after mashing had no to little effects on the precursors.[17,18] Another study evaluated the malting process as a promising tool to alter the precursor contents.[15] This assumption could be confirmed as the use of "under-modified" malts (germination time, 5 days; germination temperature, 12 °C; steeping degree, 42%) in comparison to "highly modified" malts (germination time, 7 days; germination temperature, 18 °C; steeping degree, 48%) led to a significant reduction of the cinnamic acid concentration of 56% in barley and of 30% in wheat malts.[15]

Thus, the aim of this study was to gain a deeper insight into the fate of cinnamic, *p*-coumaric, and ferulic acid during malting and their transfer into wort to develop mitigation strategies for styrene in wheat beer. Four barley and three wheat varieties were analyzed in regard to their free, soluble ester-bound, and insoluble ester-bound phenolic acid contents before and after malting and additionally in the wort produced thereof. Therefore, high-throughput methods based on stable isotope dilution assays (SIDAs) were developed.

MATERIALS AND METHODS

Grain, Malt, and Wort Samples. All grain, malt, and wort samples were kindly provided by the Chair of Brewing Science (Technical University of Berlin, Berlin, Germany) as well as the standard brewing analysis according to MEBAK[19] (results not shown) to ensure the applicability of the malts for wheat beer production. Barley varieties used in this study were Catamaran (spring barley (sp), two-rowed (2r), Franconia, Germany), Solist (sp, 2r, Franconia), Quench (sp, 2r, Denmark), and Liga (winter barley, 2r, Franconia); wheat varieties were Elixer (Upper Palatinate, Germany), Pannonia (Czech Republic), and Torp (Denmark); all harvested in 2017. Malting conditions were as follows: grain was steeped for 4 h at 16 °C in a physiological saline solution (0.9%), followed by a 20 h air rest (25 L/min, 80% humidity). This cycle was repeated with an additional 3 h wet stand and a 21 h air rest. After adjusting a steeping degree of 45%, the samples were germinated for 5 days at 18 °C. Then, germ buds from green malt were deculmed and the material sequentially kiln-dried at 50 °C for 16 h and finally at 80 °C for 4 h to a final water content of <5%. Wort was produced according to the standard mashing program of congress mash (MEBAK 4.1.4.2,[19] EBC 4.5.1),[20] as it is closest to the mashing parameters normally used for wheat beer production.

Chemicals. The following compounds were commercially available: cinnamic acid, *p*-coumaric acid, ferulic acid, formic acid, and sodium hydroxide (Sigma-Aldrich; Merck, Darmstadt, Germany); acetonitrile and methanol (Baker, Gliwice, Poland); and hydrochloric acid (Merck). All solvents were of high-performance liquid chromatography (HPLC) gradient grade, and ultrapure water was from an in-house source.

Stable Isotopically Labeled Internal Standards. The following stable isotopically labeled internal standards were commercially obtained: [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (Sigma-Aldrich).

Sample Pretreatment. Grain and malt samples (frozen at −18 °C) were ground with a centrifugal mill (12 000 rpm, sieve opening 0.5 mm; ZM 200; Retsch, Haan, Germany) prior to use and stored in wide-necked brown glass bottles at room temperature (RT).

Extraction of Phenolic Acids from Grain and Malt and Quantitation by Stable Isotope Dilution Assays (SIDAs). *Free Phenolic Acids (PAfree).* For the extraction of PAfree, ground samples (2 g) were weighed into Precellys tubes (15 mL, CK28; Bertin Technologies, Montigny-le-Bretonneux, France) and an aqueous methanol solution (5 mL, 60% vol) and the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added. Furthermore, the samples were suspended by thorough shaking (manually) and cooled to −18 °C using a fridge for 12 h. The samples were homogenized using a Precellys bead beater (6000 rpm, 3 × 45 s, break 25 s each, cooling with liquid nitrogen; Precellys Evolution; Bertin Technologies), and the extracts were allowed to rest for 1 h at RT for equilibration. After centrifugation (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR; Thermo Fisher Scientific, Dreieich, Germany), the supernatants were membrane filtered (0.45 μm, Minisart RC, hydrophilic, 15 mm; Sartorius, Göttingen, Germany) prior to analysis by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

Soluble Phenolic Acids (PAsol). For the extraction of PAsol (=sum of free and soluble ester-bound phenolic acids), hot water (2 mL, 95 °C) was added to ground grain (100 mg) or malt (200 mg) samples, followed by thorough vortexing, leading to a suspension that was constantly shaken for 1 h at 100 °C using a ThermoMixer (600 rpm; HLC HeizThermoMixer MHL23; Digital Biomedical Imaging Systems, Pforzheim, Germany). The extracts were cooled to RT in a water bath, and the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added. After vortexing the extracts for 5 min at RT for equilibration, the samples were centrifuged (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR) the first time to separate the ground samples from the liquid phase and the second time (15 000 rpm, 5 min, RT; Himac CT 15 RE; VWR, Ismaning, Germany) to remove suspended particles from the supernatant. Aliquots of these extracts (0.5 mL) were then transferred into Eppendorf vials (2 mL) and hydrolyzed in the presence of sodium hydroxide (0.5 mL, 4 mol/L) by incubation for 2 h at RT at constant shaking (600 rpm; ThermoMixer). Subsequently, the pH value was neutralized by the addition of hydrochloric acid (0.5 mL, 4 mol/L) and the samples were thoroughly vortexed and membrane filtered (0.45 μm) prior to analysis by HPLC-MS/MS.

Total Phenolic Acids (PAtot). Kim et al.[21] proved that acidic hydrolysis subsequent to alkaline hydrolysis did not increase the amounts of cinnamic, *p*-coumaric, and ferulic acid, but hot acidic conditions led to degradation. Thus, for the determination of PAtot (=sum of free, soluble ester-bound, and insoluble ester-bound phenolic acids), a single alkaline hydrolysis step was chosen for the release of ester-bound phenolic acids. Furthermore, no antioxidant, e.g., ascorbic acid with ethylenediaminetetraacetic acid (EDTA), was included in the alkaline hydrolysis, as *p*-coumaric acid and ferulic acid were found to be oxidative stable.[22] This led to the following sample preparation: sodium hydroxide (2.5 mL, 2 mol/L) and ground samples (25 mg for the quantitation of *p*-coumaric and ferulic acid and 75 mg for cinnamic acid, respectively) were weighed into glass vials (13 mL), and the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 1. Selected Ions (m/z) of Precursor and Product Ions of Phenolic Acids and the Corresponding Stable Isotopically Labeled Internal Standards, Their Retention Times, Collision Energies, Calibration Line Equations, and Coefficients of Determination (R^2) Used for Stable Isotope Dilution Assays

analyte	precursor ion (m/z)	product ions (m/z)[a]	retention time (min)	collision energies (eV)	calibration line equation[b]	R^2
cinnamic acid	149	**131**, 103, 77	9.18	8; 16; 34		
[$^2H_7$]-cinnamic acid	156	**137**, 109, 81	9.10	8; 16; 34	0.738x + 0.032	0.999
p-coumaric acid	165	**147**, 119, 91	6.75	8; 14; 22		
[$^{13}C_3$]-*p*-coumaric acid	168	**150**, 121, 93	6.77	8; 14; 22	1.090x − 0.057	0.999
ferulic acid	195	**145**, 117, 89	7.10	14; 20; 26		
[$^{13}C_3$]-ferulic acid	198	**148**, 119, 91	7.09	14; 20; 26	0.960x + 0.051	0.999

[a]Product ions in bold were used as quantifier ions and other product ions as qualifier ions. [b]Determined via a 7-point-calibration using mixtures of known amounts of unlabeled analyte and corresponding stable isotopically labeled internal standard.

(dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added. The samples were covered with argon, sealed, and suspended by thorough vortexing. The suspensions were hydrolyzed for 2 h (time optimum evaluated in a preliminary study; results not shown) at RT using a Multi-Tube Vortexer (VWR). Subsequently, hydrochloric acid (2.5 mL, 2 mol/L) was added for neutralization, and the hydrolysates were thoroughly mixed and centrifuged (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR). After membrane filtration (0.45 μm), the content of PAtot was quantitated by means of HPLC-MS/MS.

Quantitation of Phenolic Acids in Wort by Stable Isotope Dilution Assays (SIDAs). *Free Phenolic Acids.* For the quantitation of PAfree, aliquots of wort (0.8 mL) were mixed with methanol (0.8 mL), yielding samples with 50% of methanol. The addition of methanol proved to be crucial to eliminate enzymatic degradation of the precursors. After adding the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments), the samples were thoroughly mixed (GFL Orbital Shaker 3005; Gesellschaft für Labortechnik, Burgwedel, Germany) for 30 min at RT for equilibration and membrane filtered (0.45 μm) prior to HPLC-MS/MS analysis.

Soluble Phenolic Acids. PAsol were determined from hydrolyzed wort. Therefore, aliquots (0.5 mL) were spiked with the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) and hydrolyzed by the addition of a sodium hydroxide solution (0.5 mL, 4 mol/L), followed by an incubation time of 2 h at RT with constant shaking (600 rpm; ThermoMixer). After incubation, the hydrolysates were neutralized by the addition of hydrochloric acid (0.5 mL, 4 mol/L), and the samples were thoroughly vortexed, membrane filtered (0.45 μm), and analyzed by HPLC-MS/MS.

Quantitation of Phenolic Acids by High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS). HPLC-MS/MS analysis was performed using an UltiMate 3000 HPLC system (Thermo Scientific; Dionex Softron, Germering, Germany) equipped with a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific, Bremen, Germany). For the separation of the precursor acids, a Kinetex C18 column (100 × 2.1 mm, 2.6 μm, 10.0 nm; Phenomenex, Aschaffenburg, Germany) was applied using the following conditions: solvent A, aqueous formic acid (FA) (0.1% (v/v)), solvent B, FA in acetonitrile (0.1% (v/v)); gradient: 0−2 min, 10% B; 2−15 min, from 10 to 90% B; 15−18 min, 90% B; 18−19 min, from 90 to 10% B; 19−30 min, 10% B; flow gradient: 0−1 min, 0.05 mL/min; 1−2 min, from 0.05 to 0.2 mL/min; 2−30 min, 0.2 mL/min; injection volume: 10−20 μL; and column temperature: 24 °C. The ion source was operated in positive atmospheric pressure chemical ionization ($APCI^+$) mode using the following parameters: discharge current, 4.0 μA; vaporizer temperature, 250 °C; sheath gas pressure, 30 arbitrary units (au); auxiliary gas pressure, 10 au; declustering voltage, −10 V; and capillary temperature, 300 °C. Multiple reaction monitoring (MRM) was used to analyze the transitions from precursor to product ions applying experimentally optimized collision energies.

Calibration lines were determined from mixtures of known amounts of the unlabeled analyte and the corresponding stable isotopically labeled internal standard in 7 different concentration ratios (10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10) with good linearities (R^2 > 0.99) for all analytes in the applied ranges (Table 1).

Method Development for the Quantitation of Free Phenolic Acids in Grain and Malt. Based on the method of Langos et al.[16] (method A) for the determination of free phenolic acids in grain and malt, a high-throughput extraction method was developed. First, the original second extraction step was skipped (single extraction method (method B)). To check whether this had an impact on the analytical parameters, such as the limit of detection (LoD), the limit of quantitation (LoQ), or the recovery rates, both methods were compared. In addition, a liquid homogenization procedure in combination with a bead beater (method C) was tested, including a method validation for its suitability as a high-throughput method.

Quantitation of Free Phenolic Acids. According to Langos et al.,[16] method A was performed as follows: methanol (200 mL) and the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added to the ground samples (10 g). Free phenolic acids were extracted for 200 min at constant stirring (RT), and then the samples were sedimented and filtered (Folded Filter, 5 H/N, 185 mm, 85 g/m^2; Sartorius). The solvent was removed using a rotary evaporator (40 °C, 150 mbar), the residue was dissolved in water (20 mL) by ultrasonication for 5 min at RT, and the solution was extracted with ethyl acetate (4 × 20 mL). After drying the combined organic phases over anhydrous sodium sulfate and subsequent filtration, the solvent was again evaporated to dryness (30 °C, 140 mbar). The residue was dissolved in water/acetonitrile (1 mL; 9 + 1, v + v; 5 min ultrasonication at RT), and the samples were membrane filtered (0.45 μm) prior to HPLC-MS/MS analysis.

Method B included the following steps: methanol (200 mL) and the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added to the ground samples (10 g), and free phenolic acids were extracted for 200 min at constant stirring (RT). Subsequently, the samples were sedimented and filtered (Folded Filter, 5 H/N, 185 mm, 85 g/m^2), and the solvent was removed using a rotary evaporator (40 °C, 150 mbar). The residues were dissolved in water/acetonitrile (1 mL; 9 + 1, v + v; 5 min ultrasonication at RT), and the samples were membrane filtered (0.45 μm) prior to HPLC-MS/MS analysis.

Method C is described in the paragraph "Extraction of Phenolic Acids from Grain and Malt and Quantitation by Stable Isotope Dilution Assays (SIDAs)".

LoD and LoQ. LoD and LoQ of free phenolic acids were estimated from a pale wheat malt, analyzed according to the abovementioned methods, based on a signal-to-noise ratio of 3:1 and 10:1,[23] respectively.

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Recovery Rates. Recovery rates were determined using a representative pale wheat malt. Therefore, triplicates of this malt were analyzed once without the addition of the analytes and once spiked with the analytes cinnamic, *p*-coumaric, and ferulic acid, doubling the amount of the precursors originally found in the pale wheat malt. After sample work-up, according to the abovementioned methods, free phenolic acids were quantitated by means of HPLC-MS/MS and recovery rates were calculated for each analyte.

Determination of Dry Mass (dm) of Grain and Malt. Dry mass (dm) contents of grain and malts of barley and wheat were determined according to the EBC method 2.5.1.1.[20]

Statistical Analysis. Means and standard deviations for all data were calculated using Microsoft Office Excel 2007 (Microsoft Corporation, Seattle, WA).

RESULTS AND DISCUSSION

Development of the Stable Isotope Dilution Assays (SIDAs). Individual solutions of cinnamic acid, *p*-coumaric acid, and ferulic acid as well as of [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid were prepared in H_2O + ACN (9 + 1, v + v) and full MS scans were measured with both positive and negative electrospray ionization ($ESI^{+/-}$) as well as positive and negative atmospheric pressure chemical ionization ($APCI^{+/-}$). Additionally, also the collision energies were varied during full MS scans aimed at not only finding the best ionization method at particular energy but also finding the overall best ionization method. Based on the full MS scans, $APCI^+$ was proven to be the most appropriate ionization method showing the highest intensities for the analytes and standards. Next, these individual solutions were analyzed by MS/MS to select the respective precursor ions and determine the collision energies necessary for the highest yields of the three major product ions (Table 1). Four HPLC columns, Kinetex C18, Synergi Polar-RP, Synergi Hydro-RP, and Synergi Fusion-RP (all Phenomenex), were tested for the separation of these phenolic acids, proving the Kinetex C18 column as the best choice. A typical chromatogram obtained during the quantitation of these phenolic acids is exemplarily shown for a wheat malt sample in Figure 1. Identification was based on the parameters retention time, m/z of the precursor ion, and m/z of the three product ions using MRM, and the developed method provided sufficient selectivity and sensitivity in grain and malts of barley and wheat.

Development of a High-Throughput Extraction Method for Free Phenolic Acids in Grain and Malt. In the literature, plenty of studies can be found, dealing with the quantitation of phenolic acids in grain and malt. Thereby, different extraction solvents, such as pure water,[24] aqueous buffer solutions or water/organic solvent mixtures,[25−29] or pure organic solvents,[30,31] as well as different extraction times between 2 and 90 min were applied. Furthermore, phenolic acids were mainly determined by HPLC in combination with a diode array detector,[12,25,27,28,30,32,33] a CoulArray detector,[22,24] or a mass selective detector.[34,35] However, validation of the extraction efficiency of the methods was scarcely performed. Addressing this deficiency, Langos et al.[16] studied the impact of different solvents and extraction times on the extraction efficiency of phenolic acids, showing that the use of pure water was insufficient, most likely due to the still active enzymes during extraction. In contrast, methanol, with its ability to inactivate enzymes, was proven to be the best extraction solvent. Additionally, Langos et al.[16] developed SIDAs based on HPLC-MS/MS to consider polarity and possible instabilities of phenolic acids during work-up. However, this

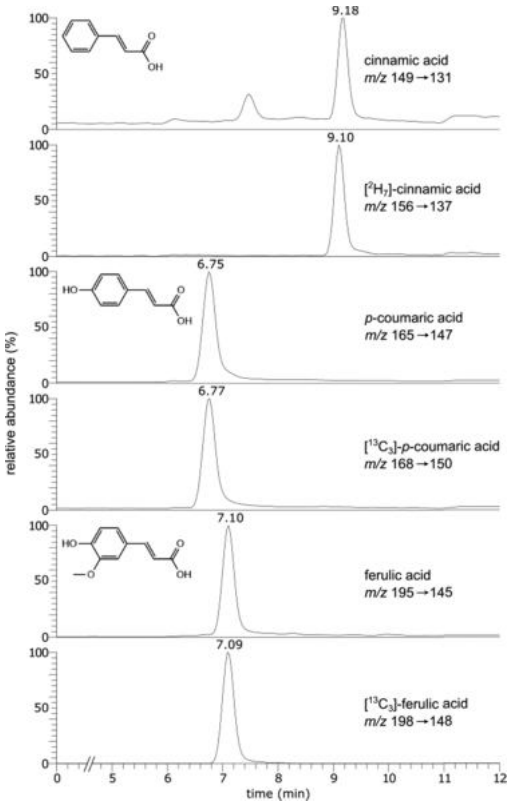


Figure 1. HPLC-MS/MS ($APCI^+$) chromatogram obtained during quantitation of free phenolic acids in a wheat malt sample by stable isotope dilution analysis showing the MRM transitions from the precursor to the product ions used for quantitation.

method (A), consisting of two extraction steps, is very time-consuming. Thus, the first aim of the present study was the development of a high-throughput method for the quantitation of free phenolic acids in grain and malt. Therefore, a single extraction method (B), which skipped the second extraction step of the original method, and a liquid homogenization procedure in combination with a bead beater (C) were tested for their suitability.

The analysis of a representative wheat malt, once with the original extraction method by Langos et al. (A) and once with the newly developed single extraction method (B), showed an accordance of the determined concentrations of 108% for cinnamic acid, 103% for *p*-coumaric acid, and 83% for ferulic acid. However, the analyte areas of method A showed high variations (rel. standard deviation (rel. SD) 75−110%) and the area counts were only 27−54% compared to method B, representing more stable area counts (rel. SD 1−7%). In fact, in both cases, the internal standards corrected the variations, but B showed higher area counts as well as a higher reproducibility. Furthermore, validation of B revealed its suitability for the determination of free phenolic acids in grain and malt with excellent recovery rates of 103% for cinnamic acid, 104% for *p*-coumaric acid, and 101% for ferulic acid (Table 2). Thereby, it was crucial to add methanol prior to the addition of the stable isotopically labeled internal standards to the samples, as otherwise bad recovery rates were

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 2. Limit of Detection (LoD), Limit of Quantitation (LoQ), and Recovery Rates of Cinnamic, *p*-Coumaric, and Ferulic Acid Determined in Wheat Malt by either the Double Extraction Method (A) According to Langos et al.,[16] the Single Extraction Method (B), or the Liquid Homogenization Method in Combination with a Bead Beater (C). Data Published by Langos et al.[16] Are Given for Comparison

	cinnamic acid[a]			*p*-coumaric acid[a]			ferulic acid[a]		
extraction method	LoD (μg/kg dry mass)	LoQ (μg/kg dry mass)	recovery rate (%)	LoD (μg/kg dry mass)	LoQ (μg/kg dry mass)	recovery rate (%)	LoD (μg/kg dry mass)	LoQ (μg/kg dry mass)	recovery rate (%)
Langos et al.[16]	30	100	99	30	100	103	10	33	97
A	6.6	22	nd[b]	0.7	2.2	nd[b]	0.3	1.1	nd[b]
B	11	37	103	2.8	9.3	104	23	75	101
C	3.1	10	105	0.8	2.8	105	3.0	9.9	101

[a]Mean values of triplicates. [b]Not determined.

obtained, due to remaining enzyme activities. Regarding LoDs and LoQs, a higher matrix in the case of method B, as a result of the omission of the second extraction step, led to higher values compared to method A (Table 2). Nevertheless, both methods had lower LoDs and LoQs in the present study using a different mass spectrometry setup, except for ferulic acid in the case of method B, as compared to the values reported by Langos et al.[16] Therefore, the single extraction method is suitable to analyze free phenolic acid contents in grain and malt with a reduced sample preparation time.

The gained knowledge from B proved that a single methanol extraction is sufficient and opened the way to use another sample extraction technique with methanol as the only solvent. Based on the fact that also the new method B is still time-consuming, due to the solvent removal after methanol extraction by rotary evaporation, a liquid homogenization in combination with a bead beater was tested as a high-throughput method for the determination of free phenolic acids in grain and malt (C). This technique is characterized by the possibility to grind samples directly within the extraction solvent and by the use of high sample amounts compared to the extraction solvent, allowing the quantitation of free phenolic acids directly from the extracts via HPLC-MS/MS. However, the first trial resulted in chromatograms with bad peak shapes using extracts obtained with 100% methanol. A stepwise dilution of such an extract with distilled water, leading to methanol contents between 80 and 20%, revealed 60% methanol to be the maximum content at which no negative effects on the chromatographic separation performance was found. As Langos et al.[16] proved, distinct amounts of methanol are necessary to inhibit enzymatic activities during phenolic acid extraction. A comparison of a wheat malt sample obtained by the use of 100 and 60% methanol (according to C) showed that the phenolic acid contents were approximately 20% higher if 60% methanol was used for extraction compared to 100% methanol. An enzymatic degradation of these acids within 100% of methanol could be excluded, as spiking experiments with cinnamic, *p*-coumaric, and ferulic acid revealed recovery rates of approximately 100%. Mechanical stress by bead beating had generally no effect on the phenolic acid contents, proven by recovery rates of 95 to 100% for a wheat malt sample, analyzed with 100% methanol using methods B and C. Additionally, an enzymatic release in the case of 60% methanol could be excluded, as phenolic acid concentrations remained constant over a storage time of 7 days (94 to 99% of phenolic acids were found compared to day 0). However, in the presence of 40% water, a better wetting and swelling of the ground wheat malt sample was visually observed. Therefore, it was assumed that this led to a better extractability of the target analytes, which resulted in the higher amounts found with 60% methanol as the extraction solvent and, thus, in a more accurate result.

Again, a method validation was performed revealing the recovery rates of 105% for cinnamic and *p*-coumaric acid and 101% for ferulic acid, proving the suitability of the developed method (C) for the determination of free phenolic acids in grain and malt (Table 2). Additionally, LoDs and LoQs could be lowered by a factor of 3 to 8 in comparison to method B, yielding 3.1 and 10 μg/kg dm for cinnamic acid, 0.8 and 2.8 μg/kg dm for *p*-coumaric acid, and 3.0 and 9.9 μg/kg dm for ferulic acid, respectively (Table 2).

Malting- and Mashing-Dependent Changes in the Amounts of Free, Soluble, and Total Cinnamic, *p*-Coumaric, and Ferulic Acid. Phenolic acids, introduced into the brewing process, are originally bound to arabinoxylan,[28,30,36] an important nonstarch polysaccharide and cell wall building block in grain. Arabinoxylans are straight sugar chains of xylose, with single irregularly attached arabinose units.[37] These straight polysaccharide chains are linked via phenolic acids, bound to the arabinose moieties, to a three-dimensional network.[38,39] As a consequence of the enzyme activity during malting, this three-dimensional network is degraded. This depletion is accompanied by the release of phenolic acids, which might then be existent in either a free, soluble ester-bound, or insoluble ester-bound form, depending on the length of the attached sugar chain. According to the preserved enzyme activity after malting, a further breakdown with a further release of the different phenolic acid forms can be assumed during mashing. Up to now, studies investigating the coherence between vinyl aromatics and their precursors in wheat beer have only been focused on the free form of phenolic acids.[10−12,14−18] However, no correlation was found between the free precursor concentrations in any of the processing steps of wheat beer production compared to the final vinyl aromatic concentrations in wheat beer.[17,18] The reason therefore is given by the fact that the feruloyl esterase is still active after kiln-drying[40] and that yeast has its own feruloyl esterase.[41] Therefore, the parameter of interest is the sum of soluble phenolic acids, which can be transferred into wort, rather than only the free form. Thus, the next aims of this study were (i) to analyze the contents of the different forms of phenolic acids in grain, malt, and wort, (ii) how malting and mashing affected these contents, (iii) and which coherence is given between the ratios of the different forms and between the different phenolic acids within these processing steps.

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 3. Free, Soluble (sol; Sum of Free and Soluble Ester-Bound), and Total (Sum of Free, Soluble Ester-Bound, and Insoluble Ester-Bound) Concentrations of Cinnamic, *p*-Coumaric, and Ferulic Acid in 4 Barley (B) and 3 Wheat (W) Varieties as well as in Malts and Wort Produced Thereof

			concentration (mg/kg dry mass)[a]						
phenolic acid	type		Catamaran (B)	Solist (B)	Quench (B)	Liga (B)	Elixer (W)	Pannonia (W)	Torp (W)
cinnamic acid	free	grain	0.049 (±35)	0.052 (±49)	0.055 (±24)	0.031 (±9)	<LoQ[b]	0.033 (±4)	<LoQ[b]
		malt	0.447 (±19)	0.492 (±17)	0.606 (±15)	0.352 (±24)	0.383 (±10)	0.344 (±20)	0.544 (±14)
		wort[c]	1.49 (±18)	1.60 (±8)	1.73 (±6)	1.36 (±9)	1.03 (±10)	1.05 (±6)	1.28 (±7)
	sol	grain	0.252 (±33)	0.202 (±34)	0.269 (±28)	0.121 (±8)	0.311 (±5)	0.243 (±4)	0.371 (±4)
		malt	1.01 (±15)	1.18 (±9)	1.15 (±2)	0.970 (±10)	0.799 (±8)	0.686 (±9)	1.01 (±9)
		wort[c]	1.55 (±3)	1.71 (±10)	1.76 (±8)	1.29 (±7)	1.26 (±3)	1.51 (±1)	1.46 (±3)
	total	grain	0.384 (±4)	0.330 (±16)	0.378 (±4)	0.332 (±9)	0.325 (±5)	0.257 (±15)	0.380 (±11)
		malt	1.26 (±8)	1.27 (±0.3)	1.12 (±9)	0.960 (±13)	1.05 (±29)	0.861 (±12)	1.16 (±5)
p-coumaric acid	free	grain	1.73 (±1)	1.23 (±2)	3.27 (±2)	2.29 (±3)	0.127 (±3)	0.159 (±2)	0.251 (±5)
		malt	0.982 (±10)	0.845 (±9)	0.790 (±19)	1.02 (±7)	0.965 (±6)	0.610 (±12)	0.978 (±8)
		wort[c]	13.5 (±7)	9.43 (±3)	8.82 (±5)	12.6 (±5)	1.03 (±24)	1.35 (±14)	2.90 (±12)
	sol	grain	10.6 (±5)	7.69 (±4)	11.0 (±2)	10.5 (±4)	2.52 (±1)	3.45 (±5)	3.28 (±5)
		malt	10.0 (±2)	8.43 (±2)	7.60 (±1)	9.73 (±5)	4.98 (±2)	4.67 (±3)	4.93 (±1)
		wort[c]	17.3 (±11)	10.3 (±5)	8.99 (±7)	13.7 (±8)	1.34 (±7)	2.08 (±4)	3.22 (±3)
	total	grain	211 (±5)	236 (±7)	306 (±4)	417 (±3)	23.7 (±5)	25.1 (±5)	27.9 (±6)
		malt	221 (±2)	244 (±6)	340 (±12)	419 (±5)	40.0 (±3)	35.8 (±7)	40.1 (±3)
ferulic acid	free	grain	8.16 (±1)	4.95 (±2)	9.16 (±5)	7.01 (±4)	5.71 (±2)	6.40 (±3)	7.04 (±2)
		malt	8.61 (±5)	8.20 (±7)	7.75 (±4)	8.62 (±8)	8.81 (±3)	7.27 (±4)	8.83 (±4)
		wort[c]	6.72 (±21)	1.41 (±33)	18.6 (±7)	2.70 (±5)	27.8 (±12)	20.9 (±8)	25.6 (±6)
	sol	grain	125 (±4)	93.6 (±1)	90.6 (±1)	104 (±3)	161 (±5)	165 (±4)	151 (±5)
		malt	165 (±2)	141 (±2)	116 (±1)	160 (±2)	168 (±1)	173 (±2)	142 (±2)
		wort[c]	85.5 (±1)	70.7 (±1)	79.9 (±2)	72.0 (±1)	120 (±3)	111 (±3)	102 (±1)
	total	grain	537 (±5)	421 (±1)	423 (±8)	561 (±6)	449 (±5)	484 (±3)	556 (±7)
		malt	1800 (±6)	1560 (±6)	1490 (±6)	1930 (±6)	1730 (±7)	1800 (±7)	1690 (±5)

[a]Mean values of triplicates with relative standard deviations (%) in parentheses (technical $n = 3$ (barley), $n = 4$ (wheat); analytical $n = 3$). [b]LoQ = 10 μg/kg dm. [c]Phenolic acid concentrations were related to the amount of malt (50 g), which was used for the production of wort (400 mL) by congress mashing.

Table 4. Factors Representing the Content Changes of the Different Phenolic Acid Forms during Malting and Mashing. Factors >1 Led to an Increase and <1 to a Decrease

			factor						
			Catamaran	Solist	Quench	Liga	Elixer	Pannonia	Torp
cinnamic acid	malting	total	3.3	3.9	3.0	2.9	3.2	3.3	3.1
		soluble	4.0	5.8	4.3	8.0	2.6	2.8	2.7
		free	9.2	9.5	11	12	nc[a]	10	nc[a]
	mashing	soluble	1.5	1.4	1.5	1.3	1.6	2.2	1.4
		free	3.3	3.2	2.9	3.9	2.7	3.1	2.4
p-coumaric acid	malting	total	1.0	1.0	1.1	1.0	1.7	1.4	1.4
		soluble	0.9	1.1	0.7	0.9	2.0	1.4	1.5
		free	0.6	0.7	0.2	0.4	7.6	3.8	3.9
	mashing	soluble	1.7	1.2	1.2	1.4	0.3	0.4	0.7
		free	14	11	11	12	1.1	2.2	3.0
ferulic acid	malting	total	3.4	3.7	3.5	3.4	3.9	3.7	3.0
		soluble	1.3	1.5	1.3	1.5	1.0	1.0	0.9
		free	1.1	1.7	0.8	1.2	1.5	1.1	1.3
	mashing	soluble	0.5	0.5	0.7	0.4	0.7	0.6	0.7
		free	0.8	0.2	2.4	0.3	3.2	2.9	2.9

[a]Not calculable.

To the best of our knowledge, this is the first time that the total and soluble amounts of cinnamic acid were quantitated in the mentioned brewing steps. The amounts of the total, soluble, and free phenolic acids in grain, malt, and wort of 4 barley and 3 wheat varieties are summarized in Table 3. In the case of cinnamic acid, it was proven that all three forms were highly affected by malting and mashing, as the total, soluble, and free cinnamic acid contents constantly increased from grain via malt to wort by factors between 1.3 and 12 (Table 4). Taking into account that the total cinnamic acid contents increased from grain to malt by factors of 2.9−3.9 (malting, total) and from malt to wort by factors of 1.3−2.2 (mashing, soluble), malting should have a stronger impact on the maximum cinnamic acid content, highlighting the importance

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

for a deeper understanding of the malting process. Regarding the amounts of cinnamic acid, independently of the phenolic acid form and the processing step, barley varieties always showed slightly higher contents compared to wheat varieties (Table 3). However, these differences, e.g., in the concentrations of soluble cinnamic acid in the wort of barley and wheat were very low, with concentrations for barley varieties of 1.29−1.76 mg/kg dm and 1.26−1.51 mg/kg dm for wheat varieties. Therefore, no significant reduction of styrene in wheat beer can be expected using different ratios of barley and wheat malts, and a reduction by the use of different varieties also seems to be very limited. Within the present study, a reduction of cinnamic acid by the selection of a variety with a low content of the undesired precursor can be assumed to be <30%.

Different from cinnamic acid, total *p*-coumaric acid was only slightly affected by malting. In the case of barley varieties, the total contents did not change upon malting and for the wheat varieties, an increase by 40−70% was found. Although malting and mashing had some decreasing and increasing effects on the free and soluble forms of *p*-coumaric acid in barley and wheat (Tables 3 and 4), these effects were negligible considering the huge concentration differences of total *p*-coumaric acid between unmalted barley (211−417 mg/kg dm) and unmalted wheat (23.7−27.9 mg/kg dm), which had a direct impact on the amount of soluble and free *p*-coumaric acid, with always higher contents for the barley varieties (Table 3). Of further interest was the huge increase of free *p*-coumaric acid during mashing in the barley varieties by factors of 11−14 (Table 4), which might have been the result of high feruloyl esterase activities in the barley varieties.

Comparable amounts of the total ferulic acid content in grain were found among the barley and wheat varieties (421−561 mg/kg dm; Table 3). This was similar to cinnamic acid, for which also no significant concentration differences were present. Malting, again similar to cinnamic acid, led to a strong increase of the total ferulic acid content by factors of 3.0−3.9. However, and now different from cinnamic acid, the soluble and free forms of ferulic acid in the wort were not really affected (Tables 3 and 4). Regarding the impact of mashing, soluble ferulic acid contents decreased similarly in the barley and wheat varieties by 30−60%, with overall higher contents in wheat wort (Table 3). This data fits with the knowledge of brewers that higher contents of wheat malt used in wheat beer production lead to a stronger wheat beer aroma. Langos et al.[18] confirmed this empiric knowledge, demonstrating that wheat beers brewed with 100% of wheat malt compared to beers brewed with 100% of barley malt had significantly higher contents of 2-methoxy-4-vinylphenol, a key odorant for the typical wheat beer aroma. This was interesting since the corresponding barley malts showed higher contents of free ferulic acid compared to the wheat malts.[18] According to our data, in the case of ferulic acid, the different forms showed no correlation between the malt and wort. However, the wort produced of barley malt had significantly lower contents of soluble ferulic acid with 70.7−85.5 mg/kg dm compared to wheat malts with 102−120 mg/kg dm (Table 3), which explains the higher contents of 2-methoxy-4-vinylphenol in wheat beer produced with higher amounts of wheat malt.

The increase of total cinnamic, *p*-coumaric (except for the barley varieties), and ferulic acid during malting (Table 4) was not expected, as the biosynthesis of these compounds to link the arabinoxylan chains does not make sense in regard to the breakdown of this structure during malting. However, this neglects the fact that cinnamic, *p*-coumaric, and ferulic acid, as part of the phenylpropanoid metabolism (Figure 2), are

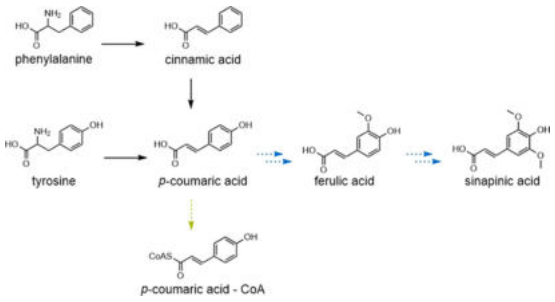


Figure 2. Biosynthesis of phenolic acids via the phenylpropanoid metabolism. Starting from phenylalanine and tyrosine, *p*-coumaric acid is either directly formed by deamination or via cinnamic acid with an additional hydroxylation step. Beginning with *p*-coumaric acid, two biosynthetic pathways start, the lignin (in blue) and the flavonoid (in green) pathway.

needed for the formation of secondary plant metabolites, such as lignin, flavonoids, and stilbenoids.[42,43] Therefore, the increase of the total amount of phenolic acids is suggested to be a consequence of the need for the seedling of these secondary plant metabolites during germination. In fact, this approach can also explain the decrease of free *p*-coumaric acid during malting for all barley varieties (Table 4). Further, the total and soluble (not significant) *p*-coumaric acid contents were not increasing during malting of barley varieties, which might suggest that the biosynthesis of *p*-coumaric acid is slower compared to the conversion of *p*-coumaric acid within further biosynthetic pathways.

Effect of Malting and Mashing on the Ratio of Free, Soluble Ester-Bound, and Insoluble Ester-Bound Phenolic Acids. A comparison of the total amount of phenolic acids in malt with the soluble amount in wort (Table 3) revealed transfer rates for cinnamic acid from 124 to 175%, for *p*-coumaric acid from 2.6 to 8.0%, and for ferulic acid from 3.7 to 6.9% (Table 5). Transfer rates of >100% for cinnamic acid from malt into wort cannot be explained by a complete transfer of the already free and soluble ester-bound forms (cf. Quench and Liga; Figure 3) in combination with further enzymatic activities during mashing solubilizing remaining contents of insoluble ester-bound cinnamic acid. Thus, an additional biosynthesis of cinnamic acid during mashing must have taken place. In the case of *p*-coumaric and ferulic acid, transfer rates of <8% revealed that for the desired precursors, the predominant form in malt was the insoluble ester-bound one and, furthermore, that the activities of enzymes involved in the degradation process were too low to release higher amounts of insoluble precursors present in malt.

In detail, already in grain, cinnamic acid occurred mainly in a soluble form with >61% in barley (with the exception of Liga (36%)) and >95% in wheat (Figure 3). By malting, enzymatic activities in barley varieties led to an increase of the soluble forms. In the case of Quench and Liga, already 100% of cinnamic acid were present in a soluble form. Interestingly, in the case of wheat, a decrease of the soluble form took place during malting. However, these still high percentages of the soluble forms in malts were only found for cinnamic acid and

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 5. Transfer Rates of the Total Cinnamic, *p*-Coumaric, and Ferulic Acid Contents from Malt into Wort

	transfer rate (%)						
	Catamaran	Solist	Quench	Liga	Elixer	Pannonia	Torp
cinnamic acid	124	134	157	134	120	175	125
p-coumaric acid	7.8	4.2	2.6	3.3	3.4	5.8	8.0
ferulic acid	4.7	4.5	5.4	3.7	6.9	6.2	6.0

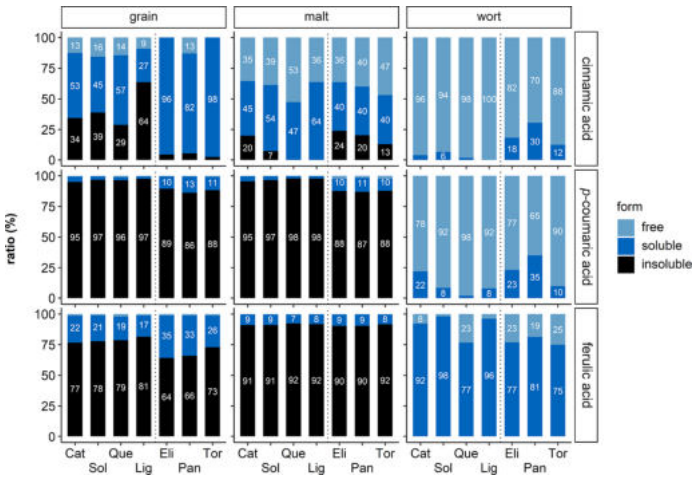


Figure 3. Ratio of free, soluble ester-bound, and insoluble ester-bound cinnamic, *p*-coumaric, and ferulic acid in grain, malt, and wort of the barley varieties Catamaran (Cat), Solist (Sol), Quench (Que), and Liga (Lig) and of the wheat varieties Elixer (Eli), Pannonia (Pan), and Torp (Tor).

not for the desired precursors. The occurrence of cinnamic acid predominantly in a soluble form in grain and malt can be ascribed to the role of cinnamic acid in the phenylpropanoid metabolism (Figure 2), in which it is only an intermediate between L-phenylalanine and *p*-coumaric acid.[42,43] After mashing, the free form of cinnamic acid was the most dominant one in the wort. Already 94−100% of soluble cinnamic acid in barley wort and 70−88% in wheat wort were present in the free form (Figure 3). From the studies of Langos et al.,[17,18] it is known that after fermentation, no remaining free cinnamic acid was found. Combining these results with the fact that the total amount of cinnamic acid is transferred from malt into wort and that mostly its free form is present in the wort, a complete decarboxylation of cinnamic acid should take place during fermentation. According to this consideration, it was assumed that the formed amount of styrene should directly be correlated to the concentration of its precursor. However, taking the concentrations of soluble cinnamic acid in the wort (1.29−1.76 mg/kg, Table 3), process-related dilutions, and typical styrene concentrations in wheat beers (9.8−32 μg/L)[44] into account, only approximately 20% of the formed styrene are found in a wheat beer with an original gravity of 12%. Most likely, styrene is lost due to evaporation, which has already been given as a reason for the much lower styrene contents found in wheat beers prepared via an "open fermentation" compared to a "closed fermentation".[10] Furthermore, the 4 barley and 3 wheat varieties showed a very narrow concentration range of soluble cinnamic acid in the wort, which matches the narrow distribution of the styrene concentrations (9.8−32 μg/L) found in 18 commercial wheat beers analyzed in a recent market survey,[44] and which has also already been reported by Langos et al.[17] (15−33 μg/L) and Daly et al.[2] (25−31 μg/L). Thus, it is not expected that the styrene content in commercially available wheat beers will clearly exceed the maximum content of around 33 μg/L found in these studies.

Independent of the barley and wheat varieties, the predominant form of *p*-coumaric acid in grain was the insoluble ester-bound form with 96−97% for the barley varieties and 88−89% for the wheat varieties (Figure 3). Interestingly, malting did not alter the ratios of the different forms, except for the free form in wheat malt with a slight increase. In fact, this reflected the negligible impact of the malting process on the contents of the different forms of *p*-coumaric acid in barley and on the small increase in wheat, which was similar for the ester-bound forms but more pronounced for the free form (Table 3). Additionally, the occurrence of *p*-coumaric acid in malt predominantly as an insoluble ester-bound form explains the low transfer rates of <8% during mashing. In contrast, an opposite situation was found in the wort of barley and wheat, where, comparable to cinnamic acid, *p*-coumaric acid was mostly present in its free form and only <35% was still soluble ester-bound (Figure 3). Taking the differences in the amounts of free and soluble ester-bound *p*-coumaric acid in barley malt (free: 0.79−1.02 mg/kg dm, soluble ester-bound: 7.60−10.0 mg/kg dm) and wort (free: 8.82−13.5 mg/kg dm, soluble ester-bound: 8.99−17.3 mg/kg dm) into account (Table 3), it is shown that the feruloyl esterase activity during mashing played a more important role for the final amount of *p*-coumaric acid in the wort compared to the release and transfer of the soluble ester-bound form. Similar results were obtained for the analyzed wheat varieties.

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 6. Ratio between Cinnamic, *p*-Coumaric, and Ferulic Acid in Grain, Malt, and Wort Based on the Free, Soluble (Sum of Free and Soluble Ester-Bound), and Total (Sum of Free, Soluble Ester-Bound, and Insoluble Ester-Bound) Concentrations of the Phenolic Acids

		ratio (%)							
		grain			malt			wort[a]	
phenolic acid	variety	free	soluble	total	free	soluble	total	free	soluble
cinnamic acid	Catamaran	0.5	0.2	0.1	4.5	0.6	0.1	6.9	1.5
	Solist	0.8	0.2	0.1	5.2	0.8	0.1	12.9	2.1
	Quench	0.4	0.3	0.1	6.6	0.9	0.1	5.9	1.9
	Liga	0.3	0.1	<0.1[b]	3.5	0.6	<0.1[c]	8.2	1.5
	Elixer	nc[d]	0.2	0.1	3.8	0.5	0.1	3.4	1.0
	Pannonia	0.5	0.1	0.1	4.2	0.4	<0.1[e]	4.5	1.3
	Torp	nc[d]	0.2	0.1	5.3	0.7	0.1	4.3	1.4
p-coumaric acid	Catamaran	17.4	7.8	28.2	9.8	5.7	10.9	62.2	16.6
	Solist	19.7	7.6	35.9	8.9	5.6	13.5	75.8	12.5
	Quench	26.2	10.8	42.0	8.6	6.1	18.6	30.3	9.9
	Liga	24.5	9.2	42.6	10.2	5.7	17.8	75.6	15.7
	Elixer	2.2	1.5	5.0	9.5	2.9	2.3	3.4	1.1
	Pannonia	2.4	2.0	4.9	7.4	2.6	1.9	5.8	1.8
	Torp	3.4	2.1	4.8	9.4	3.3	2.3	9.7	3.0
ferulic acid	Catamaran	82.1	92.0	71.8	85.8	93.7	89.0	31.0	81.9
	Solist	79.4	92.2	64.0	86.0	93.6	86.4	11.3	85.5
	Quench	73.4	88.9	58.0	84.7	93.0	81.4	63.8	88.1
	Liga	75.1	90.7	57.3	86.3	93.7	82.1	16.2	82.8
	Elixer	97.8	98.3	94.9	86.7	96.7	97.7	93.1	97.9
	Pannonia	97.1	97.8	95.0	88.4	97.0	98.0	89.7	96.9
	Torp	96.6	97.6	95.2	85.3	96.0	97.6	86.0	95.6

[a]The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort. [b]0.03%. [c]0.04%. [d]Not calculable, due to content <LoD. [e]0.05%.

Also, ferulic acid, similar to *p*-coumaric acid, occurred mainly in the insoluble ester-bound form in grain and malt. In grain, up to 81% of ferulic acid in barley and wheat were insoluble ester-bound, with even higher ratios of up to 92% in malt (Figure 3). A comparison of the different forms of ferulic acid in the wort revealed that for barley only 2−8% (with the exception of Quench (23%)) of the soluble forms were present already in the free form and for the wheat wort, the ratio was higher with 19−25% (Figure 3). In fact, this is different from cinnamic and *p*-coumaric acid for which nearly all of the soluble form was present in its free form. This fact is related to the significantly higher amount of the soluble fraction of ferulic acid in the wort that has to be released by the feruloyl esterase. Additionally, it is consistent with the study by Schwarz et al.,[13] showing that free ferulic acid constantly increased over 6 h in the wort with a steep slope as a function of increasing mashing-in time at 45 °C, whereas the content of *p*-coumaric and cinnamic acid increased at a very flat rate and seemed to reach a plateau very early in the mashing process.

Taking into account that the soluble phenolic acids in the wort already consisted of 70 to 100% of free cinnamic acid, 65−98% of free *p*-coumaric acid, and only 2−25% of free ferulic acid (Figure 3), in combination with the huge concentration differences between the phenolic acids (Table 3), it can be assumed that during fermentation, maximum styrene concentrations are reached much faster compared to those of 4-vinylphenol and 2-methoxy-4-vinylphenol. The time to reach the maximum amount of 2-methoxy-4-vinylphenol should be the highest, as yeasts have to not only convert a higher amount of free ferulic acid but also release ferulic acid from soluble ester-bound forms. This assumption is corroborated by the results of Schwarz et al.,[10,11] who monitored the styrene and 2-methoxy-4-vinylphenol contents during wheat beer fermentation. For styrene, maximum contents were already found after 2 h, whereas for 2-methoxy-4-vinylphenol maximum contents were reached after 6 days at a fermentation temperature of 16 °C and after 3 days at 25 °C.

Influence of Malting and Mashing on the Ratio of Cinnamic, *p*-Coumaric, and Ferulic Acid. Mitigation strategies for styrene on the basis of malting and mashing aimed at the reduction of cinnamic acid, while relevant concentrations of the desired phenolic acids should be maintained. Therefore, the ratio between cinnamic, *p*-coumaric, and ferulic acid is an important information to evaluate the relevance of mitigation strategies. Table 6 summarizes the ratio of the precursors in the processing steps based on the different phenolic acid forms. It can be concluded that the total cinnamic acid content made up ≤ 0.1% in grain and malt, independent of barley and wheat. In contrast, *p*-coumaric and ferulic acid showed huge differences in grain of barley and wheat varieties. In barley, both ferulic and *p*-coumaric acid contributed to nearly half of the total amount each, whereas in wheat, ferulic acid made up around 95% and *p*-coumaric acid only around 5%. In fact, this is an interesting difference between barley and wheat varieties, which can be found at all stages of the brewing process (Table 3). During malting of wheat, the amounts of phenolic acids increased in general; however, the ratios remained unaffected, with around 98% of ferulic acid, 2% of *p*-coumaric acid, and <1% of cinnamic acid, due to comparable degrees of increase (Table 4). For barley varieties, only the contents of cinnamic acid and ferulic acid increased, while the *p*-coumaric acid content did not change (Table 4), resulting in the composition

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

of approximately 85% of ferulic acid, 15% of *p*-coumaric acid, and <1% of cinnamic acid.

Fortunately, the total cinnamic acid concentrations (0.86−1.27 mg/kg dm) were very low in the malts compared to those of ferulic acid (1490−1930 mg/kg dm) and *p*-coumaric acid (221−419 mg/kg dm (barley), 35.8−40.1 mg/kg dm (wheat)). This explains another fact that was found by Langos et al.,[17] who monitored a high release of the free desired precursors during mashing compared to free undesired cinnamic acid, although, for example, free *p*-coumaric and cinnamic acid contents were equal in malt. This observation can be addressed to the very low total contents of cinnamic acid.

For the wheat varieties, also mashing affected all three precursors similarly, whereupon only minor changes were found in the ratios between the soluble phenolic acids in the wort compared to malt, as ferulic acid accounted for 96−98%, *p*-coumaric acid for 1.1−3.0%, and cinnamic acid for 1.0−1.4%. Also, for the barley varieties, mashing had a minor effect on the ratios of soluble phenolic acids, with percentages in the wort of 82−88% for ferulic acid, 10−17% for *p*-coumaric acid, and 1.5−2.1% for cinnamic acid (Table 6). Interestingly, in the case of the free form in the wort, *p*-coumaric acid made up the biggest part within the barely varieties with a percentage of 62−76%, except for Quench with 30% (Table 6). This can be explained by the incomplete transfer of free ferulic acid during mashing, which was the only phenolic acid with a rate of <100%. Langos et al.[17] reported that phenolic acids are unstable during sample extraction in the presence of 100% of water. They described the mashing process, in principal an aqueous extraction, as a very dynamic process consisting of different degrees of degradation of free phenolic acids and a release of bound phenolic acids. Based on this fact, the degradation of free ferulic acid was stronger than the release of bound ferulic acid for barley in the present study. Taking these results together, the use of barley malts led to an introduction of cinnamic, *p*-coumaric, and ferulic acid into the wort in a ratio of 2:14:84, and the use of wheat malt in a ratio of 1:2:97, respectively.

These results confirmed previously published data of *p*-coumaric and ferulic acid in grain, malt, and wort. For example, Anderson et al.[45] found free ferulic acid contents in 7 barley varieties between 1.4 and 5.9 mg/kg dm, soluble ester-bound ferulic acid contents between 21.7 and 42.5 mg/kg dm, and insoluble ester-bound ferulic acid contents between 104 and 365 mg/kg dm. For *p*-coumaric acid, content ranges were 0.6−7.0 mg/kg dm, 1.7−13.1 mg/kg dm, and 2.7−109 mg/kg dm for the free, soluble ester-bound, and insoluble ester-bound forms. The fact that all of these forms of ferulic acid were found at a factor of 2 higher within the present study can be addressed to the different analyzed barley varieties. Based on these new data, clear differences in the compositions of the precursors have to be expected within different barley varieties. Additionally, Li et al.[46] examined 130 winter and 20 spring wheat varieties and found free, soluble ester-bound, and insoluble ester-bound ferulic acid contents of 1.2−6.2 mg/kg dm, 9.4−62.4 mg/kg dm, and 162−721 mg/kg dm and *p*-coumaric acid contents of 0−2.2 mg/kg dm, 3.0−14.6 mg/kg dm, and 2.8−19.1 mg/kg dm, respectively. Except for the total *p*-coumaric acid amount, with slightly higher contents, the wheat varieties analyzed in this study were in a similar range as reported by Li et al.[46] Total *p*-coumaric and ferulic acid contents in malts of the present study were further in accordance with the results of Vanbeneden et al.,[47] who found in 9 barley varieties contents between 204 and 360 mg/kg dm for *p*-coumaric acid and between 481 and 653 mg/kg dm for ferulic acid. In addition, they compared the total amounts of phenolic acids in malt with the total amounts in wort and reported on similar transfer rates between 4 and 5% for *p*-coumaric acid and between 7 and 13% for ferulic acid compared to those of the present study. Furthermore, the wort analyzed in the study of Vanbeneden et al. also showed similar contents of soluble *p*-coumaric acid, with similar proportions of the free form (54−81%) compared to those found in the present study. However, proportions of free ferulic acid (19−43%) were generally higher compared to those of the present study (2−8%), except for Quench with 23% of free ferulic acid as the soluble form in the wort. A comparison of the contents of soluble ferulic acid reveals similar amounts in both studies, whereas for free ferulic acid, lower amounts were found in the present study. An explanation for this discrepancy might be the used quantitation methods for free phenolic acids in the wort. In both methods, quantitation was performed directly after membrane filtration (0.45 μm, regenerated cellulose), with the distinction that in the method used in the present study, the wort was first mixed with methanol in a ratio of 1 + 1 (v + v), which was proven to be crucial to inactivate enzyme activities. Very interestingly, experiments without the addition of methanol during the method development for free phenolic acids in the wort in the present study revealed that in the case of barley, only ferulic acid, and in the case of wheat, only *p*-coumaric acid, showed a degradation, suggesting that the involved grain-specific enzymes are highly selective. Additionally, the lack of stable isotopically labeled internal standards in the study of Vanbeneden et al.[47] may contribute to the differences in the quantitated amounts.

In summary, changes in the contents of the different phenolic acid forms of cinnamic, *p*-coumaric, and ferulic acid, and coupled to this, changes in the ratios of the different forms as well as the ratios between the desired and undesired precursors, are the result of the contributions of the biosynthesis of the phenolic acids, their use within further catabolic pathways, the degradation of arabinoxylan, and the direct release of phenolic acids by the feruloyl esterase to different extents. To the best of our knowledge, this is the first study reporting on the quantitation of the total and soluble cinnamic acid contents in grain, malt, and wort, and additionally constantly monitored free, soluble ester-bound, and insoluble ester-bound cinnamic, *p*-coumaric, and ferulic acid in these samples. The new insights into the malting process corroborated its suitability for mitigation strategies of the toxicologically relevant styrene, whereupon different malting steps should be monitored in more detail to gain more knowledge about their ability to alter the cinnamic acid content in malt, with the consequence of an overall lower styrene content in wheat beer, still eliciting the desired aroma notes.

AUTHOR INFORMATION

Corresponding Author

Michael Granvogl − *Institut für Lebensmittelchemie, Fachgebiet Lebensmittelchemie und Analytische Chemie (170a), Fakultät Naturwissenschaften, Universität Hohenheim, D-70599 Stuttgart, Germany;* orcid.org/0000-0003-1281-9999; Phone: +49 0711 459 23979; Email: michael.granvogl@uni-hohenheim.de; Fax: +49 0711 459 24096

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

Authors

Valerian Kalb − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany*

Torsten Seewald − *Institut für Lebensmitteltechnologie und Lebensmittelchemie, Fachgebiet Brauwesen, Technische Universität Berlin, D-13353 Berlin, Germany*

Thomas Hofmann − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany;* orcid.org/0000-0003-4057-7165

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c04835

Funding

The IGF Project (AiF 19593 N) of the Association for the Promotion of Science of the German Brewing Industry (Wifoe) was supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a decision by the German Bundestag.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Miss Anneliese Köhler for technical assistance and Mr. Sami Kaviani-Nejad for performing the LC-MS/MS measurements.

■ REFERENCES

(1) Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. *J. Agric. Food Chem.* **2013**, *47*, 11303−11311.

(2) Daly, B.; Collins, F.; Madigan, D.; Donelly, D.; Coakley, M.; Ross, P. An investigation into styrene in beer. *Proc. Congr. - Eur. Brew. Conv.* **1997**, *26*, 623−630.

(3) Goodey, A. R.; Tubb, R. S. Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *J. Gen. Microbiol.* **1982**, *128*, 2615−2610.

(4) Renner, R.; Bartels, H.; Tressl, R. Enrichment and quantitative determination of beer aroma substances by gas extraction (in German). *Monatsschr. Brau.* **1976**, *29*, 478−480.

(5) Tressl, R.; Friese, L.; Fendesack, F.; Koeppler, H. Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. *J. Agric. Food Chem.* **1978**, *6*, 1422−1426.

(6) WHO. *Guidelines for Drinking-Water Quality: Health Criteria and Other Supporting Information*, 2nd ed.; World Health Organization: Geneva, Switzerland, 1996.

(7) IARC. *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, Some Mycotoxins, Naphtalene and Styrene*; International Agency for Research on Cancer: Lyon, France, 2002.

(8) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicol. Sci.* **1998**, *46*, 266−281.

(9) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Bevan, C.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J. Appl. Toxicol.* **2001**, *21*, 185−198.

(10) Schwarz, K. J.; Stübner, R.; Methner, F.-J. Formation of styrene dependent on fermentation management during wheat beer production. *Food Chem.* **2012**, *4*, 2121−2125.

(11) Schwarz, K. J.; Boitz, L. I.; Methner, F.-J. Enzymatic formation of styrene during wheat beer fermentation is dependent on pitching rate and cinnamic acid content. *J. Inst. Brew.* **2012**, *3*, 280−284.

(12) Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Release of phenolic acids and amino acids during mashing dependent on temperature, pH, time and raw materials. *J. Am. Soc. Brew. Chem.* **2012**, *70*, 290−295.

(13) Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Influence of mashing conditions on the release of precursors of phenolic wheat beer aroma (in German). *Brauwelt* **2013**, *153*, 274−279.

(14) Schwarz, K.; Methner, F.-J. Styrene concentrations during wheat beer production. *Brew. Sci.* **2011**, *64*, 156−158.

(15) Langos, D.; Granvogl, M.; Gastl, M.; Schieberle, P. Influence of malt modifications on the concentrations of free phenolic acids in wheat and barley malts. *Brew. Sci.* **2015**, *68*, 93−101.

(16) Langos, D.; Granvogl, M.; Meitinger, M.; Schieberle, P. Development of stable isotope dilution assays for the quantitation of free phenolic acids in wheat and barley and malts produced thereof. *Eur. Food Res. Technol.* **2015**, *5*, 637−645.

(17) Langos, D.; Granvogl, M. Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. *J. Agric. Food Chem.* **2016**, *11*, 2325−2332.

(18) Langos, D.; Gastl, M.; Granvogl, M. Reduction of toxicologically relevant styrene in wheat beer using specially produced wheat and barley malts. *Eur. Food Res. Technol.* **2017**, *193*, 558−568.

(19) Pfenninger, H. *Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommision (MEBAK)*, 3rd ed.; Brautechnische Analysenmethoden 1; Selbstverlag der MEBAK: Freising, Germany, 1997.

(20) European Brewery Convention. *Analytica—EBC*, 9th ed.; Fachverlag Hans Carl: Nuremberg, Germany, 2010.

(21) Kim, K.-H.; Tsao, R.; Yang, R.; Cui, S. W. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chem.* **2006**, *3*, 466−473.

(22) Nardini, M.; Ghiselli, A. Determination of free and bound phenolic acids in beer. *Food Chem.* **2004**, *1*, 137−143.

(23) U.S. Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry, Q2B Validation of Analyical Procedures: Methodology*; Center for Drug Evaluation and Research: Rockville, MD, 1996.

(24) Dvořáková, M.; Douanier, M.; Jurková, M.; Kellner, V.; Dostálek, P. Comparison of antioxidant activity of barley (*Hordeum vulgare* L.) and malt extracts with the content of free phenolic compounds measured by high performance liquid chromatography coupled with CoulArray detector. *J. Inst. Brew.* **2008**, *2*, 150−159.

(25) Woffenden, H. M.; Ames, J. M.; Chandra, S.; Anese, M.; Nicoli, M. C. Effect of kilning on the antioxidant and pro-oxidant activities of pale malts. *J. Agric. Food Chem.* **2002**, *17*, 4925−4933.

(26) Samaras, T. S.; Camburn, P. A.; Chandra, S. X.; Gordon, M. H.; Ames, J. M. Antioxidant properties of kilned and roasted malts. *J. Agric. Food Chem.* **2005**, *20*, 8068−8074.

(27) Inns, E. L.; Buggey, L. A.; Booer, C.; Nursten, H. E.; Ames, J. M. Effect of heat treatment on the antioxidant activity, color, and free phenolic acid profile of malt. *J. Agric. Food Chem.* **2007**, *16*, 6539−6546.

(28) Dvořáková, M.; Guido, L. F.; Dostálek, P.; Skulilová, Z.; Moreira, M. M.; Barros, A. A. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *J. Inst. Brew.* **2008**, *1*, 27−33.

(29) Inns, E. L.; Buggey, L. A.; Booer, C.; Nursten, H. E.; Ames, J. M. Effect of modification of the kilning regimen on levels of free ferulic acid and antioxidant activity in malt. *J. Agric. Food Chem.* **2011**, *17*, 9335−9343.

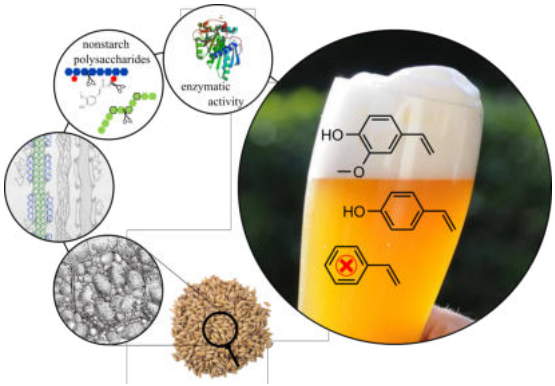
(30) Maillard, M.-N.; Berset, C. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. *J. Agric. Food Chem.* **1995**, *7*, 1789−1793.

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

(31) Maillard, M.-N.; Soum, M.-H.; Boivin, P.; Berset, C. Antioxidant activity of barley and malt: relationship with phenolic content. *LWT - Food Sci. Technol.* **1996**, *29*, 238−244.
(32) Dvořáková, M.; Hulin, P.; Karabín, M.; Dostálek, P. Determination of polyphenols in beer by an effective method based on solid-phase extraction and high performance liquid chromatography with diode-array detection. *Czech J. Food Sci.* **2007**, *4*, 182−188.
(33) Zhao, H.; Chen, W.; Lu, J.; Zhao, M. Phenolic profiles and antioxidant activities of commercial beers. *Food Chem.* **2010**, *3*, 1150−1158.
(34) Lu, Y.; Luthria, D.; Fuerst, E. P.; Kiszonas, A. M.; Yu, L.; Morris, C. F. Effect of processing on phenolic composition of dough and bread fractions made from refined and whole wheat flour of three wheat varieties. *J. Agric. Food Chem.* **2014**, *43*, 10431−10436.
(35) Nicoletti, I.; Martini, D.; de Rossi, A.; Taddei, F.; D'Egidio, M. G.; Corradini, D. Identification and quantification of soluble free, soluble conjugated, and insoluble bound phenolic acids in durum wheat (*Triticum turgidum* L. var. *durum*) and derived products by RP-HPLC on a semimicro separation scale. *J. Agric. Food Chem.* **2013**, *48*, 11800−11807.
(36) Nordkvist, E.; Salomonsson, A.-C.; Åman, P. Distribution of insoluble bound phenolic acids in barley grain. *J. Sci. Food Agric.* **1984**, *35*, 657−661.
(37) Fincher, G. B. Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. *Plant Physiol.* **2009**, *1*, 27−37.
(38) Fincher, G. B. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 305−346.
(39) Gubler, F.; Ashford, A. E. Release of ferulic acid esters from barley aleurone. I. Time course of gibberellic-acid-induced release from isolated layers. *Aust. J. Plant Physiol.* **1985**, *3*, 297−305.
(40) Humberstone, F. J.; Briggs, D. E. Extraction and assay of ferulic acid esterase from malted barley. *J. Inst. Brew.* **2000**, *1*, 21−29.
(41) Coghe, S.; Benoot, K.; Delvaux, F.; Vanderhaegen, B.; Delvaux, F. R. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **2004**, *3*, 602−608.
(42) Hahlbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347−369.
(43) Ververidis, F.; Trantas, E.; Douglas, C.; Vollmer, G.; Kretzschmar, G.; Panopoulos, N. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. *Biotechnol. J.* **2007**, *10*, 1214−1234.
(44) Kalb, V.; Granvogl, M. Formation of Desired Smoky Key Odorants in Wheat Beer: a Comparison with the Undesired Toxicologically Relevant Styrene. In *Sex, Smoke, and Spirits: The Role of Chemistry*; ACS Symposium Series 1321; Guthrie, B., Beauchamp, J. D., Buettner, A., Toth, S., Qian, M. C., Eds.; American Chemical Society: Washington, DC, 2019; pp 93−105.
(45) Andersson, A. A. M.; Lampi, A.-M.; Nyström, L.; Piironen, V.; Li, L.; Ward, J. L.; Gebruers, K.; Courtin, C. M.; Delcour, J. A.; Boros, D.; Fraś, A.; Dynkowska, W.; Rakszegi, M.; Bedo, Z.; Shewry, P. R.; Åman, P. Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.* **2008**, *21*, 9767−9776.
(46) Li, L.; Shewry, P. R.; Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.* **2008**, *21*, 9732−9739.
(47) Vanbeneden, N.; Gils, F.; Delvaux, F.; Delvaux, F. R. Variability in the release of free and bound hydroxycinnamic acids from diverse malted barley (*Hordeum vulgare* L.) cultivars during wort production. *J. Agric. Food Chem.* **2007**, *26*, 11002−11010.

https://dx.doi.org/10.1021/acs.jafc.0c04835
J. Agric. Food Chem. 2020, 68, 12421−12432

3.3 The Role of Endogenous Enzymes during Malting of Barley and Wheat Varieties in the Mitigation of Styrene in Wheat Beer



3.3.1 Summary

Insoluble ester-bound, soluble ester-bound, and free forms of phenolic acids are a direct consequence of the degradation processes occurring during germination (part of the malting process). With the objective of getting a deeper insight into the enzyme activities, directly or indirectly involved in the release of phenolic acids during malting, grain and malts of different barley and wheat varieties were analysed in this study for their α- and β-amaylase, β-glucanase, feruloyl esterase, and protease activities. Additionally, aiming at a more detailed picture of the involved biochemical processes, also the determination of the contents of the nonstarch polysaccharides β-glucan and especially of total and water-extractable arabinoxylan to which phenolic acids are preliminary bound was included into this study.

In grain, β-glucan contents were much higher for the barley varieties compared to the wheat varieties. Upon malting, a strong degradation of β-glucan occurred, leading to similar low amounts in both grain types. Since β-glucan is incorporated into the cell walls in form of bundles, the nearly complete degradation of this nonstarch polysaccharide structure was synonymous to a depletion of the cell walls. As further also arabinoxylan is a major building block of cell walls, this suggested that phenolic acids in malts were not trapped in intact cell walls. Having a look at the activities of the corresponding enzyme β-glucanase, responsible for the degradation of β-glucan, a reverse effect was found upon malting, starting with very low activities in the grain and ending up with very high activities in the malt. Further, for the barley varieties, high β-glucan contents in the grain were found to correlate with high β-glucanase activities in the malts, which was vice versa for the wheat varieties. In fact, this seems to reflect the adaption of these two grain types to their β-glucan contents, guaranteeing the accessibility of starch as an energy source for the seedling by an efficient depletion of the cell walls.
Grain of barley and wheat showed comparable amounts of total arabinoxylan and upon malting,

no significant changes were found. This was different for the water-extractable arabinoxylan content, with clearly higher contents in grain of the wheat varieties compared to the barley varieties. Malting led to an increase of the water-extractable contents in both cases, however, with still higher amounts in the wheat malts. Additionally higher soluble ferulic acid contents in wheat wort compared to barley wort, prepared from the same malts, indicated that the degradation of the nonstarch polysaccharide arabinoxylan has an impact on the release of phenolic acids.
Feruloyl esterase activities, capable of the direct release of phenolic acids from arabinoxylan, proved to be slightly higher in grain of the wheat varieties compared to the barley varieties. During malting, in both grain types, the feruloyl esterase activity increased, however, with now higher activities in the malts of the barley varieties. With significantly higher feruloyl esterase activities in barley malts and at the same time comparable total cinnamic and ferulic acid contents in malts of both grain types, barley malts were expected to show higher free phenolic acid contents. As this was not the case, it was assumed that the complex interaction between enzyme activities, inhibitor proteins, phenolic acid contents, etc. in the respective varieties overlapped the impact of the feruloyl esterase activity.
α- and β-Amylase activities are not directly linked to the release of phenolic acids, however, are of general interest for beer brewing due to their role in the saccharification of starch. With activities in line with data from literature, the applicability of the prepared malts of this study for wheat beer brewing was verified.
By comparison of the protease activity to the feruloyl esterase, α- and β-amlyase, and β-glucanase activities in grain and malts of the barley and wheat varieties, no correlation was found, highlighting that enzymes, directly or indirectly involved in the release of phenolic acid, were unaffected by the protease activity.

The individual contributions of V. Kalb and the co-authors' contributions are specified below:

V. Kalb designed the study, developed the enzymatic assays, and conducted the laboratory work. Grain and malt samples were supplied by T. Seewald, Chair of Brewing Science and Beverage Technology, Technische Universität Berlin. A. Köhler, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, assisted in sample preparation. V. Kalb statistically analysed and interpreted the data, discussed the results, and created the figures and tables. He developed the story of the paper, wrote the first draft of the manuscript, and revised the paper. T. Hofmann, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, coordinated the research project. Prof. Dr. M. Granvogl, Institute of Food Chemistry, University of Hohenheim, was responsible for the conceptualization, project administration, supervision of the research project, and writing reviews and editing.

3.3.2 Publication

The Role of Endogenous Enzymes during Malting of Barley and Wheat Varieties in the Mitigation of Styrene in Wheat Beer

V. Kalb, T. Seewald, T. Hofmann, and M. Granvogl

Permanent weblink: https://dx.doi.org/10.1021/acs.jafc.0c04837

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

The Role of Endogenous Enzymes during Malting of Barley and Wheat Varieties in the Mitigation of Styrene in Wheat Beer

Valerian Kalb, Torsten Seewald, Thomas Hofmann, and Michael Granvogl*

Cite This: J. Agric. Food Chem. 2020, 68, 13888−13896 Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Knowledge of the biochemical processes responsible for the release of phenolic acids (precursors of vinyl aromatics) during malting is important to find mitigation strategies for the toxicologically relevant styrene (formed from cinnamic acid) in wheat beer. Therefore, grain and malts of four barley and three wheat varieties were screened for the activities of various enzymes and the amounts of nonstarch polysaccharides (to which the phenolic acids are bound to a certain extent). During malting, a very strong degradation of β-glucan, synonymous to a depletion of the cell walls, was found, suggesting that a partial degradation of cell walls cannot have an effect on the release of phenolic acids. In barley malts, water-extractable arabinoxylan contents were between 0.59 and 0.79 g/100 g dm and in wheat malts between 0.93 and 1.51 g/100 g dm. Additionally, higher soluble ferulic acid contents in wheat wort compared to barley wort indicated that the degradation of nonstarch polysaccharides has an impact on the release of phenolic acids. For the feruloyl esterase, higher activities were found in malts of the barley varieties. However, this fact was not reflected by the free phenolic acid contents in those malts. Correlation coefficients between the protease activity and the feruloyl esterase, α- and β-amylase, and β-glucanase activities were proven to be insignificant, highlighting that the protease activity had no effect on the activities of these other enzymes.

KEYWORDS: *styrene, phenolic acids, feruloyl esterase, wheat beer, malt, wort, enzymatic assays*

INTRODUCTION

Styrene, well-known for its use as a monomer in the plastic industry, is also present in several groceries. This is also the case for wheat beer, which has to be produced with at least 50% of wheat malt and in which styrene was detected already in the 1970s.[1,2] However, not much attention was paid to this fact until 1996 when the World Health Organization (WHO) published a tolerable daily intake (TDI) of 7.7 μg/kg body weight per day based on the classification of styrene as a toxicant.[3] Following the studies on rats[4] and mice,[5] the toxicological assessment of styrene was updated by the International Agency for Research on Cancer (IARC) in 2002, classifying styrene as possibly carcinogenic to humans (class 2B).[6] As a consequence, mitigation strategies for foods containing styrene had to be developed. However, the difficulty of this objective is given by the structural relationship between styrene and two key aroma compounds of wheat beer, namely, 4-vinylphenol and 2-methoxy-4-vinylphenol. These two odorants are responsible for the formation of the characteristic aroma of wheat beer,[7] which is described as clove-like and slightly phenolic. All three vinyl aromatics derive from the same precursor class of cinnamic acid derivatives, traditionally also referred to as phenolic acids, with cinnamic acid as the precursor of styrene, *p*-coumaric acid of 4-vinylphenol, and ferulic acid of 2-methoxy-4-vinylphenol. These precursors are initially bound to arabinoxylan,[32−34] an important nonstarch polysaccharide in malts of barley and wheat. Together with β-glucan, an additional nonstarch polysaccharide, arabinoxylan is a major building block of the cell walls of cereals. Arabinoxylans consist of straight sugar chains of xylose, with single irregularly attached arabinose units.[35] These straight polysaccharide chains are linked to a three-dimensional network by bridges built up from phenolic acids, bound to the arabinose moieties.[36,37] Into this gel-like network, bundles of β-glucan are incorporated, responsible for the stiffness of the cell walls.[38]

The conversion of these phenolic acids into the corresponding vinyl aromatics during the brewing process is mainly enzymatically driven[8] and occurs during fermentation in the presence of top-fermenting, Pof$^+$-active yeasts.[9,10] This relationship explains why, in most strategies, focusing on mashing[11] and fermentation,[9] a reduction of styrene was accompanied by a reduction of the desired vinyl aromatics, leading to wheat beers without or at least with a lowered impression of the typical aroma expected by consumers.

A different approach to mitigate styrene in wheat beer is based on a reduction of its precursor cinnamic acid during malting. A study performed by Langos et al.[12] confirmed that malting is a promising tool to alter the precursor contents. The use of "undermodified" malts (steeping degree, 42%; germination temperature, 12 °C; germination time, 5 days)

Received: July 28, 2020
Revised: September 28, 2020
Accepted: October 6, 2020
Published: November 5, 2020

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

in comparison to "highly modified" malts (steeping degree, 48%; germination temperature, 18 °C; germination time, 7 days) led to a significant reduction of cinnamic acid in wheat malts by 30% and in barley malts by 56%, whereas the contents of the desired phenolic acids remained high enough for a well-pronounced aroma release. A very recent study reported on the amounts of free, soluble ester-bound, and unsoluble ester-bound cinnamic, *p*-coumaric, and ferulic acid and discussed the fate of these precursors during malting and how they were transferred into wort.[13] However, up to now, the impact of endogenous enzymes, such as α- and β-amylase, β-glucanase, feruloyl esterase, and proteases, on the release of phenolic acids during malting is not perfectly understood, e.g., their activities in grain and malts.

Thus, the aim of the present study was to get a deeper insight into the enzyme activities during malting that influence directly or indirectly the release of phenolic acids. Therefore, grain and malts of four barley and three wheat varieties were screened for the activities of α- and β-amylase, β-glucanase, feruloyl esterase, and proteases. Additionally, total and water-extractable arabinoxylan contents as well as β-glucan contents were analyzed due to the fact that the target phenolic acids are preliminary bound to arabinoxylan.

MATERIALS AND METHODS

Grain and Malt Samples. Grain and malt samples of the barley varieties Catamaran (spring barley (sp), two-rowed (2r), Franconia, Germany), Solist (sp, 2r, Franconia), Quench (sp, 2r, Denmark), and Liga (winter barley, 2r, Franconia), and the wheat varieties Elixer (Upper Palatinate, Germany), Pannonia (Czech Republic), and Torp (Denmark), all harvested in 2017, were kindly supplied by the Chair of Brewing Science (Technical University of Berlin, Berlin, Germany). It was also ensured that malts were applicable for wheat beer production using the standard brewing analysis according to MEBAK.[14] Malting conditions were as follows: steeping: 4 h wet stand at 16 °C (physiological saline solution (0.9%)); 20 h air rest, 25 L/min (80% humidity); 3 h wet stand at 16 °C; 21 h air rest, 25 mL/min; steeping degree: 45%; germination: 5 days at 18 °C; deculming; kiln-drying: 16 h at 50 °C and 4 h at 80 °C; water content: < 5%.

Chemicals. The following compounds were commercially available: acetic acid, calcium chloride dihydrate, ethanol, hydrochloric acid, sodium acetate, sodium chloride, trichloroacetic acid, and zinc acetate dihydrate (Merck, Darmstadt, Germany); azo-casein, L-cysteine hydrochloride monohydrate, DL-malic acid, and sodium hydroxide (Sigma-Aldrich Chemie, Taufkirchen, Germany); 2-morpholinoethanesulfonic acid (Mes) and polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100) (AppliChem, Darmstadt); 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris base) and *p*-nitrophenol (Carl Roth, Karlsruhe, Germany); bovine serum albumin and ethylenediaminetetraacetic acid (EDTA) (Serva, Heidelberg, Germany); 4-nitrophenyl *trans*-ferulate (Carbosynth, Compton, Berkshire, United Kingdom); and sodium dihydrogen phosphate dihydrate (Fluka, Neu-Ulm, Germany).

Sample Pretreatment. Grain and malt samples were frozen at −18 °C prior to grinding with a centrifugal mill (12,000 rpm; sieve opening, 0.5 mm; ZM 200; Retsch, Haan, Germany). Ground samples were stored in wide-necked brown glass bottles at room temperature (RT).

Quantitation of Nonstarch Polysaccharides in Grain and Malts. *Total Arabinoxylan Contents.* Contents were determined by means of a D-xylose assay kit (K-XYLOSE 04/16; Megazyme, Wicklow, Ireland). Briefly, ground samples (100 mg) were weighed into glass vials (13 mL), hydrochloric acid (5 mL; 1.3 mol/L) was added, and the samples were shaken (600 rpm) for 1 h at 100 °C for hydrolysis using a ThermoMixer (HLC HeizThermoMixer MHL23; Digital Biomedical Imaging Systems, Pforzheim, Germany). The hydrolysates were cooled to RT in a water bath, the pH value was neutralized by the addition of sodium hydroxide solution (5 mL; 1.3 mol/L), and the samples were quantitatively transferred into volumetric flasks (100 mL). After adjusting the volume with distilled water and thorough manual mixing, aliquots were transferred into polypropylene vials (15 mL) and centrifuged (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR; Thermo Fisher Scientific, Dreieich, Germany). The supernatants were further used in the enzyme assay.

For the enzyme assay, distilled water (550 μL) and aliquots of the supernatants of the hydrolysates (500 μL) were filled into semi-micro cuvettes (PMMA, 1.6 mL, 1 cm light path; Ratiolab, Dreieich) followed by the addition of the buffer (200 μL; pH 7.5, 0.02% (w/v) of sodium azide), the NAD^+/ATP solution (200 μL), and the hexokinase suspension (10 μL). After thorough mixing by tilting the cuvettes and incubation for 5 min at 25 °C, the absorbance was detected via a UV-Vis spectrophotometer (UV-2401 PC; Shimadzu, Duisburg, Germany) at 340 nm (A1). In the second step, a xylose mutarotase/β-xylose dehydrogenase solution (25 μL) was added, the mixtures were again thoroughly mixed by tilting the cuvettes, incubated for 6 min at 25 °C, and the absorbance was detected again (A2). Additionally, a blank sample with distilled water was treated in the same way (B1, B2). For each sample set, a D-xylose standard solution (0.25 mg/mL) was used as a control. The D-xylose content was calculated from the difference of A2 to A1 and subtraction of the difference in blank absorption (B2-B1) via the Lambert-Beer law with a molar extinction coefficient for NADH of 6300 L/(mol·cm). From the resulting D-xylose contents in grain and malts, the amounts of arabinoxylan were calculated considering the condensation factor of 0.88 and the D-xylose content of arabinoxylan in barley and wheat, which was specified to be 62%.[15,16]

Water-Extractable Arabinoxylan Contents. Contents were determined according to the abovementioned procedure for the total arabinoxylan content with a modified sample preparation. Ground samples (100 mg) were weighed into polypropylene vials (15 mL), and hot water (6.5 mL, 85 °C) was added. Subsequently, the enzymes were deactivated[17] by incubating the samples at 90 °C for 15 min at constant shaking (600 rpm) on a ThermoMixer followed by an additional extraction step[18] at 60 °C for 30 min. Then, the extracts were cooled to RT in a water bath and centrifuged (4600 rpm, 10 min, RT; Heraeus Multifuge X3 FR). The supernatants were transferred into glass vials (13 mL) and hydrochloric acid (0.943 mL; 32%) was added for hydrolysis at 100 °C for 1 h at constant shaking (600 rpm; ThermoMixer). The hydrolysates were cooled to RT in a water bath and quantitatively transferred into volumetric flasks (20 mL). After addition of sodium hydroxide solution (7.386 mL; 1.3 mol/L) for neutralization, the volume was adjusted with distilled water and the extracts were thoroughly manually mixed. Finally, aliquots of the hydrolysates (~1.5 mL) were transferred into Eppendorf tubes (1.5 mL) and centrifuged (10,000 rpm, 5 min, RT; Himac 15RE; VWR, Ismaning, Germany) to remove the precipitates. The supernatants were further used in the enzyme assay, as described for the total arabinoxylan content, with the exception that the sample volume was set to 375 μL.

β-Glucan Contents. For the determination of the total β-glucan contents, a mixed-linkage assay kit (K-BGLU 02/17; Megazyme) was used following with some modifications the procedure approved by the European Brewing Convention for barley (EBC 3.10.1)[19] and malt (EBC 4.16.1).[19] Barley and wheat samples were analyzed as follows: first, ground samples (250 mg) were weighed into polypropylene vials (15 mL) to which an aqueous ethanol solution (0.5 mL; 1 + 1, v + v) and sodium phosphate buffer (2.5 mL; 20 mmol/L, pH 6.5) were added. The samples were vortexed thoroughly, and subsequently, the samples were alternately (0.5, 1.0, and 3.5 min) incubated in a boiling water bath and vortexed to prevent caking of the gel material. Afterward, the samples were cooled to 40 °C using a water bath and distilled water (2.5 mL) was added to reduce the viscosity. Then, a lichenase solution in sodium phosphate buffer (0.1 mL; 10 U, 10 mmol/L, pH 6.5) was added and the tubes were incubated at 40 °C for 1 h with vortexing every 15 min. After incubation, distilled water was added to the barley (9.5 mL) and wheat (4.75 mL) extracts followed by vortexing and centrifugation

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

(4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR). The supernatants were further used in the enzyme assay.

For the enzyme assay, aliquots of the extracts (0.1 mL) were mixed with a β-glucosidase solution in sodium acetate buffer (0.1 mL; 0.2 U, 50 mmol/L, pH 4.0) in glass vials (13 mL). Subsequently, the enzymatic reactions were held for 15 min at 40 °C. Afterward, the glucose determination reagent GOPOD (3 mL; glucose oxidase, peroxidase, 4-aminoantipyrine, and *p*-hydroxybenzoic acid) was added. After incubation for 20 min at 40 °C, the absorbance was measured via UV-Vis spectrometry at 510 nm. β-Glucan contents were calculated based on the absorbance differences between the samples and the reaction blank. Additionally, a factor considering the conversion of the absorbance values to micrograms of glucose that was determined from the glucose standard solution (0.1 mL; 1 g/L) was applied. With each run, a barley control standard (included in the test kit) and a reagent blank with distilled water (0.1 mL), both with sodium acetate buffer (0.1 mL) and GOPOD reagent solution (3.0 mL), were prepared.

Malt samples were analyzed in analogy to the unmalted barley and wheat samples, including a further pre-sample preparation step to wash-off glucose from β-glucan degradation. Therefore, ground malt samples (500 mg) were weighed into polypropylene vials (15 mL) to which an aqueous ethanol solution (2.5 mL; 1 + 1, v + v) was added. After vortexing, the samples were incubated for 5 min using a boiling water bath, vortexed again, and another 2.5 mL of aqueous ethanol solution was added. The samples were then centrifuged (4000 rpm, 10 min, RT; Heraeus Multifuge X3 FR), and the supernatants were discarded. The pellets were resuspended in aqueous ethanol solution (5 mL; 1 + 1, v + v), scattered with a spatula, thoroughly vortexed, and centrifuged again (4000 rpm, 10 min, RT; Heraeus Multifuge X3 FR). The supernatants were once more discarded, and the pellets were resuspended in sodium phosphate buffer (2.5 mL; 20 mmol/L, pH 6.0). Starting with the lichenase incubation, the samples were further worked-up in analogy to the sample preparation for unmalted barley and wheat samples mentioned above.

Determination of Enzyme Activities in Grain and Malts. *α- and β-Amylase Activities.* Activities were analyzed according to the Ceralpha and Betamyl-3 enzymatic activity assay kit (K-MALTA 05/15; Megazyme) with some modifications for grain. Briefly, α- and β-amylase were extracted by the addition of Betamyl-3 buffer A (5 mL; 50 mmol/L Tris base, 1 mmol/L EDTA, 100 mmol/L L-cysteine, pH 8.0) to ground samples (500 mg) in polypropylene vials (15 mL). Extraction was performed for 1 h at RT by vortexing every 10 min. The extracts were centrifuged (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR), and the supernatants were further used in the α- and β-amylase assays.

For the determination of the β-amylase activity, aliquots of the extracts (0.2 mL) were additionally diluted with Betamyl-3 buffer B (4 mL; 100 mmol/L Mes, 2 mmol/L EDTA, 1 g/L bovine albumin, pH 6.2). Aliquots (0.1 mL) were transferred into glass vials (13 mL) and incubated for 5 min at 40 °C. Preheated (40 °C) Betamyl-3 substrate solution (0.1 mL; *p*-nitrophenyl-β-D-maltotrioside and thermostable β-glucosidase (50 U)) was added to start the enzymatic reactions. The mixtures were vortexed and incubated for another 10 min at 40 °C. The enzymatic reactions were stopped by the addition of the stopping reagent (1.5 mL; 1% (w/v) of Tris base solution, pH 8.5), and the absorbance was determined via UV-Vis spectrometry at 400 nm.

For the determination of the α-amylase activity in grain, the extracts were directly used for the enzyme activity assay due to the low activity of α-amylase in grain of barley and wheat. For α-amylase activity in malt, the extracts were first diluted (1 + 21, v + v) with Betamyl-3 buffer B followed by an additional dilution (1 + 16, v + v) with Ceralpha buffer A (50 mmol/L malate, 50 mmol/L sodium chloride, 2 mmol/L calcium chloride, pH 5.4). Aliquots (0.1 mL) were then transferred into glass vials (13 mL) and incubated for 5 min at 40 °C. Subsequently, preheated (40 °C) Ceralpha substrate solution (0.1 mL; blocked *p*-nitrophenyl-α-D-maltoheptaoside and thermostable α-glucosidase (125 U)) was added to start the enzymatic reactions followed by vortexing and incubation for another 10 (malt) or 20 (grain) min at 40 °C. At the end of the reaction time, the enzymatic reactions were stopped by addition of the stopping reagent (1.5 mL; 1% (w/v) of Tris base solution, pH 8.5) and the absorbance was determined via UV-Vis spectrometry at 400 nm.

To each set of assays, a standardized malt flour (included in the test kit) was added as a control as well as a reaction blank, consisting of a stopping reagent (1.5 mL), Betamyl-3 or Ceralpha substrate solution (0.1 mL), and one representative sample extract (0.1 mL) per sequence. α- and β-Amylase activities were calculated by the Lambert-Beer law with a molar extinction coefficient for *p*-nitrophenol of 18.1 L/(mmol·cm) from the sample absorbance after subtraction of the reaction blank. One unit of activity is defined as the amount of enzyme that is needed to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl-β-D-maltotrioside or *p*-nitrophenyl-α-D-maltoheptaoside in 1 min under the defined assay conditions.

β-Glucanase Activities. Activities were quantitated by means of a malt β-glucanase activity assay (K-MBGL 03/11; Megazyme). To take the low activities in grain of barley and wheat and wheat malts into account, some modifications were necessary. Ground samples (0.5 g) were weighed into polypropylene vials (15 mL). Then, β-glucanase was extracted by the addition of a sodium acetate/sodium phosphate buffer (2 mL for grain of barley and wheat and wheat malt and 8 mL for barley malt; 40 mmol/L, pH 4.6) for 15 min at RT with manual repetitive shaking. Further, the extracts were centrifuged (3500 rpm, 10 min, RT; Heraeus Multifuge X3 FR) and the supernatants were further used in the enzyme assay.

For the determination of the β-glucanase activity, aliquots of the extracts (0.25 mL) were transferred into glass vials (13 mL) and preincubated for 5 min at 30 °C. The enzymatic reaction was started by the addition of preheated (30 °C) azo-barley glucan solution (0.25 mL; 1% (w/v)), accompanied by thorough vortexing. After 30 min of incubation at 30 °C, the enzymatic reaction was stopped by the addition of a sodium acetate/zinc acetate precipitation buffer (1.5 mL; 220 mmol/L sodium acetate, 16.5 mmol/L zinc acetate, water/ethanol/methanol (300/665/35, v/v/v), pH 5.0). The stopped reactions were allowed to stand for 5 min prior to centrifugation (3500 rpm, 10 min, RT; Heraeus Multifuge X3 FR), and the absorbance was measured via UV-Vis spectrometry at 590 nm. Each set of determination included a malt flour with standardized β-glucanase activity (included in the test kit) and one reaction blank composed of the azo-barley glucan solution (0.25 mL), the precipitation solution (1.5 mL), and one representative sample extract (0.25 mL) per sequence. The β-glucanase activity was calculated from the comparison of the sample absorbance after subtraction of the reaction blank with a calibration curve, determined from the measurement of the absorbance of the dilution of a malt flour with standardized β-glucanase activity (U/kg) with a slope of 699 and an intercept of 13. The β-glucanase activity had to be corrected by the specified versus the calculated activity of the standard malt flour. One unit of enzyme activity is defined as the amount of β-glucanase that is needed to cleave 1 μmol of glycosidic linkages in 1 min under the defined assay conditions.

Feruloyl Esterase Activities. Activities in grain and malts of barley and wheat were determined according to a method based on the spectrophotometric assay of Mastihuba et al.,[20] the enzyme extraction procedure of Vanbeneden et al.,[21] and the reaction stopping approach of Großmann.[22] Therefore, ground samples (1.5 g) were suspended in Mes buffer (5 mL; 50 mmol/L Mes, pH 6.0) by thorough vortexing. Feruloyl esterase was extracted for 30 min at RT at constant shaking (900 rpm; ThermoMixer) and vortexing every 10 min. The extracts were centrifuged (4700 rpm, 15 min, RT; Heraeus Multifuge X3 FR) and membrane filtered (0.45 μm, Minisart RC, hydrophilic, 15 mm; Sartorius, Göttingen, Germany) to get a clear extract, which was further used in the enzyme assay. Prior to the assay, the freshly prepared substrate solution consisting of 4-nitrophenyl *trans*-ferulate in DMSO (10 mmol/L) was obtained by dilution (1 + 9, v + v) in potassium phosphate buffer (0.1 mol/L, 2.5% (v/v) Triton X-100, pH 6.0). For the assay, Mes buffer (0.6 mL for grain and 0.9 mL for malts) and aliquots of the extracts (0.6 mL for grain and 0.3 mL for malts) were transferred into glass vials (13 mL) and preincubated at

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

30 °C for 5 min. The enzymatic reactions were started by the addition of the substrate solution (0.4 mL) in steps of 20 s to the samples. After exactly 2 h, the reactions were stopped by the addition of the stopping reagent (3.0 mL; 1% (w/v) of Tris base in water, pH 8.5), again in steps of 20 s, and the absorbance was determined via UV-Vis spectrometry at 400 nm after 15 min after the addition of the stopping reagent to the first sample. For each triplicate, a reaction blank was performed, starting with the addition of the stopping reagent (1.5 mL), the Mes buffer (0.3 mL for grain and 0.45 mL for malts), and the extract (0.3 mL for grain and 0.15 mL for malts) into UV-Vis cuvettes (PMMA, 4 mL, 1 cm light path; Ratiolab). Then, the substrate solution was added in steps of 60 s to the reaction blanks, simulating the addition of the stopping reagent to the samples, and the reaction blanks were mixed by tilting the cuvettes. Fifteen minutes after the first addition of the substrate solution, the absorption was measured via UV-Vis spectrometry at 400 nm. The feruloyl esterase activity was calculated by comparison of the sample absorbance after subtraction of the blank absorbance with the calibration curve of 4-nitrophenol from 0.87 to 87 nmol/L in potassium phosphate buffer (0.1 mol/L, 2.5% (v/v) Triton X-100, pH 6.0). One unit of feruloyl esterase activity is defined as the amount of enzyme that is needed to release 1 nmol of 4-nitrophenol from 4-nitrophenyl *trans*-ferulate in 1 min under the defined assay conditions.

Protease Activities. Activities were analyzed, as described by Rani et al.,[23] with some modifications to simulate the conditions present in malts.[24−27] Proteases were extracted from ground samples (0.5 g) with sodium acetate buffer (2.5 mL; 50 mmol/L,[24] pH 4.9) at the pH value of malt of 4.9[25] for 1 h[26] using an ice bath[27] and vortexing every 15 min. The extracts were centrifuged (14,000 rpm, 15 min, 4 °C; Avanti J-E; Beckmann Coulter, Krefeld, Germany), and the supernatants were further used in the enzyme assay. Prior to the assay, the substrate solution was prepared by dissolving the azo-casein substrate (125 mg) in sodium acetate buffer (10 mL). Sodium acetate buffer (not for grain and 400 μL for malt) was added to the substrate solution (100 μL) and preincubated at 37 °C for 10 min in Eppendorf tubes (2 mL). The reaction was started by the addition of the extracts (600 μL for grain and 200 μL for malt) and was held for 1 h at 37 °C. Subsequently, the enzymatic reaction was terminated by adding an aqueous solution of trichloroacetic acid (0.5 mL; 10% (w/v)). The samples were allowed to rest for 15 min at 0 °C using an ice bath[28] and were further centrifuged (14,000 rpm, 10 min, 4 °C; Himac 15RE), and the supernatants were transferred into Eppendorf tubes (2 mL). A sodium hydroxide solution (40 μL; 10 mol/L) was added, the mixtures were membrane filtered (0.45 μm), and the absorbance was measured via UV-Vis spectrometry at 440 nm. For each triplicate, a reaction blank was performed with the same composition as the respective sample, but starting with mixing the aqueous solution of the stopping reagent trichloroacetic acid with the substrate solution prior to the addition of the sample extract. The enzyme activity was determined based on the difference between the absorption of the sample and the reaction blank, compared to a calibration curve of azo-casein. For the calibration curve, the absorption of azo-casein was measured in the concentration range between 4 and 605 μg/mL under assay conditions, with the exception that the aqueous solution of trichloroacetic acid was replaced by water to prevent azo-casein precipitation. One unit of enzyme activity is defined as the amount of protease releasing 1 μg of azo-casein in 1 min under the defined assay conditions.

As the activities of proteases, belonging to the class of serine proteases, strongly depend on the reducing conditions[25] present during the enzymatic reaction, protease activities were additionally determined by repeating the enzyme activity assays for proteases with the addition of cysteine (2 mmol/L) to the sodium acetate buffer (2.5 mL; 50 mmol/L, pH 4.9).

Determination of Dry Mass (dm) of Grain and Malts. Contents of the dm of grain and malts of barley and wheat were determined according to the EBC method 2.5.1.1.[19]

Statistical Analysis. Means and standard deviations for all data were calculated using Microsoft Office Excel 2007 (Microsoft Corporation, Seattle, WA), and Pearson correlation coefficients were evaluated with the programming language R (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria)[29] and the interface R Studio (version 1.1.423; RStudio, Boston, MA)[30] using the "rcorr" function implemented in the R package "Hmisc" (version 4.2.0).[31]

RESULTS AND DISCUSSION

Barley and wheat grain comprise about 75% of endosperm with the endosperm cells responsible for the storage of starch and 25% of bran[39] with the aleurone cells as important representatives, which are known to be rich in arabinoxylan.[40] According to Adom et al.,[39] ferulic acid is mostly found in the bran fraction, with contents between 1950 and 2190 mg/kg but with much lower contents in the endosperm fraction (between 29.2 and 40.8 mg/kg). In grain, arabinoxylan and β-glucans occur mainly in their intact form. However, during germination, part of the malting process, starch, as a sugar source, must be made accessible for the seedling, whereupon the cell walls of the endosperm cells have to be degraded. Additionally, also, the aleurone cells may be targeted by degrading enzymes during germination. These breakdown processes are accompanied by the release of phenolic acids. In regard to wheat beer brewing, this coherence led to the questions if (i) a partial degradation of the cell walls, (ii) the solubilization of the cell wall polysaccharides, and/or (iii) the feruloyl esterase activity have an impact on the release of either free and/or soluble ester-bound phenolic acids, and (iv) proteases affect the activity of further enzymes (α- and β-amylase, β-glucanase, and feruloyl esterase), directly or indirectly involved in the release of phenolic acids during malting.

Impact of a Possible Partial Degradation of the Cell Walls on the Release of Free or Soluble Ester-Bound Phenolic Acids. β-Glucans are incorporated in the form of bundles into the three-dimensional network of arabinoxylan.[38] A complete degradation of β-glucans by β-glucanase can only occur if these bundles are released from this network, and consequently, are accessible for the enzymes. This is only the case if the cell walls (built up by arabinoxylan and β-glucans) are degraded. Therefore, the β-glucan contents were used as an indicator for the degree of the cell wall depletion and to answer the question if a partial degradation of the cell walls plays a role in the release of phenolic acids. β-Glucan contents in the unmalted barley varieties were between 3.14 and 3.67 g/100 g dm, whereas those in the unmalted wheat varieties were only between 0.65 and 0.79 g/100 g dm (Table 1). The higher β-glucan contents in barley compared to wheat are a characteristic difference between these two types of grain.[41] During malting, a massive decrease of β-glucans was found, with similar residual contents in malts of barley (0.10−0.42 g/100 g dm) and wheat (0.25−0.31 g/100 g dm). For the corresponding enzyme β-glucanase, responsible for the depletion of β-glucans, a reverse effect was found during malting. In the case of unmalted grain, the barley varieties showed only very low, not quantifiable (<LoQ) activities, except for Solist with a β-glucanase activity of 35.7 U/kg dm, and the wheat varieties showed only very low activities between 8.1 and 8.8 U/kg dm. During malting, β-glucanase activities were strongly expressed in the barley varieties (239−337 U/kg dm), while the activities in wheat malts increased only very slightly (9.5−12.1 U/kg dm) or, in the case of Torp, even decreased marginally (from 8.4 to 8.0 U/kg dm) (Table 1).

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 1. β-Glucan Concentrations and β-Glucanase Activities in Four Barley and Three Wheat Varieties and in Malts Produced Thereof

		β-glucan concentration (g/100 g dry mass)[a]		β-glucanase activity (U/kg dry mass)[a]	
type	variety	grain	malt	grain	malt
barley	Catamaran	3.37 ± 0.11	0.15 ± 0.01	<LoQ[b]	337.1 ± 0.6
	Solist	3.28 ± 0.05	0.10 ± 0.01	35.7 ± 0.7	239.2 ± 5.5
	Quench	3.67 ± 0.02	0.42 ± 0.01	<LoQ[b]	270.3 ± 6.5
	Liga	3.14 ± 0.08	0.31 ± 0.00	<LoQ[b]	300.4 ± 1.0
wheat	Elixer	0.65 ± 0.04	0.28 ± 0.01	8.8 ± 0.2	9.5 ± 0.2
	Pannonia	0.79 ± 0.01	0.25 ± 0.01	8.1 ± 0.1	12.1 ± 0.1
	Torp	0.72 ± 0.02	0.31 ± 0.01	8.4 ± 0.1	8.0 ± 0.3

[a]Mean values of triplicates (± standard deviation). [b]LoQ = 6.8 U/kg dry mass.

Table 2. Total and Water-Extractable Arabinoxylan Concentrations in Four Barley and Three Wheat Varieties and in Malts Produced Thereof

		concentration (g/100 g dry mass)[a]			
		total arabinoxylan		water-extractable arabinoxylan	
type	variety	grain	malt	grain	malt
barley	Catamaran	6.98 ± 0.17	7.07 ± 0.28	0.27 ± 0.01	0.79 ± 0.02
	Solist	6.74 ± 0.17	7.20 ± 0.26	0.23 ± 0.01	0.66 ± 0.01
	Quench	6.41 ± 0.04	6.88 ± 0.19	0.24 ± 0.00	0.59 ± 0.01
	Liga	8.00 ± 0.23	8.10 ± 0.26	0.25 ± 0.01	0.75 ± 0.01
wheat	Elixer	6.22 ± 0.13	6.44 ± 0.15	0.67 ± 0.02	1.21 ± 0.02
	Pannonia	7.27 ± 0.09	7.43 ± 0.11	0.90 ± 0.01	1.51 ± 0.06
	Torp	6.29 ± 0.08	6.36 ± 0.07	0.54 ± 0.01	0.93 ± 0.02

[a]Data are expressed as the mean ± standard deviation (technical: $n = 3$ (barley), $n = 4$ (wheat); analytical: $n = 3$).

A comparison of the β-glucan contents to the β-glucanase activities found for the varieties investigated in the present study indicated that high β-glucanase activities in malts were always linked to high β-glucan contents in grain for the barley varieties and vice versa for the wheat varieties. This seems to reflect the adaption of the barley and wheat varieties to the present contents of β-glucan as the depletion of the cell walls has to be guaranteed for the accessibility of starch as a sugar source for the seedling during germination. Further, the very low amounts of β-glucan in barley and wheat malt varieties, produced for wheat beer brewing, indicated that a nearly complete depletion of the cell walls took place, which means that phenolic acids are not trapped in intact cell walls.

Impact of the Solubilization of Cell Wall Polysaccharides on the Release of Free or Soluble Ester-Bound Phenolic Acids. During malting, various enzymes, such as *endo*-1,4-β-xylanase, β-D-xylosidase, and α-L-arabinofuranosidase, degrade the arabinoxylan three-dimensional network into fragments of different sizes.[42] These fragments are characterized by an increased solubility in water and still contain phenolic acids, which can be transferred into wort in a soluble but still ester-bound form, whereupon they are accessible for yeast later on in the brewing process. Thus, the amount of water-extractable arabinoxylan was studied to answer the question to which extent the solubilization of the cell wall polysaccharide arabinoxylan correlates with the release of phenolic acids. Total arabinoxylan contents of the barley and wheat varieties were between 6.41 and 8.00 g/100 g dm and between 6.22 and 7.27 g/100 g dm, respectively (Table 2). During malting, no significant changes of the total arabinoxylan contents were found within the barley and wheat varieties. As the test kit determines the arabinoxylan content via free xylose after hydrolysis of the intact polysaccharide, the absence of significant differences in the total arabinoxylan content between grain and malts indicated that possibly released xylose was not prone to a further metabolism. An additional experiment on free xylose in malt (results not shown) also proved that <0.5% of the original arabinoxylan contents were degraded to the monomer xylose during malting. In contrast, the amount of water-extractable arabinoxylan (related to the total arabinoxylan content) was between 3 and 4% for the unmalted barley varieties and between 9 and 12% for the unmalted wheat varieties (Table 2). During malting, enzyme activities led to a further depletion of the arabinoxylan network, resulting in an increase of the water-extractable arabinoxylan fraction to 9−11% in the barley varieties and 15−20% in the wheat varieties. This corresponds to an increase of the water-extractable arabinoxylan fraction during malting in the barley and wheat varieties by factors of 2.5−3.0 and 1.7−1.8, respectively. Considering the comparable amounts of total arabinoxylan of the barley and wheat varieties, these results also indicated that the barley varieties had a stronger enzyme activity than the wheat varieties. A comparison of the water-extractable arabinoxylan contents of the present study to the amounts of the soluble fraction of ferulic acid in wort of the barley and wheat varieties of a very recent study[13] suggested that the degradation degree of nonstarch polysaccharides has an impact on the release of phenolic acids during the production of wheat beer. Soluble ferulic acid contents were found between 70.7 and 85.5 mg/kg dm for the barley varieties and between 102 and 120 mg/kg dm for the wheat varieties, which were similar to the results found for the water-

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

extractable arabinoxylan contents.[13] This is even more interesting as both the barley and wheat varieties showed very similar contents of total ferulic acid in malts.

Correlation between the Feruloyl Esterase Activity and the Release of Free Phenolic Acids. A preliminary study within the method development for the determination of the feruloyl esterase activity revealed (results not shown) that it was not possible to stop the enzymatic activity quantitatively by the addition of the stopping reagent, proven by a constant increase of the absorbance also after "stopping" the reaction. An activity of approximately 20% can still be expected at a pH value of 8.5, as shown by the study of Wang et al.[43] Altering the percentage of Tris base between 1, 5, and 10% (w/v) as well as the pH value of the stopping reagent between 8.5 and 10 did also not lead to a complete inhibition of the enzyme activity. Instead, a complete degradation of the substrate to 4-nitrophenol was observed at a pH value of 10.

An alternative approach to correct the activities left after "stopping" the reaction was to simulate the increase of absorption within the time between "stopping" the reaction till the measurement of the absorption using a sample blank and subtracting it from the absorption of the sample. Within the method development, it was successfully shown that this approach is sufficient to correct the remaining feruloyl esterase activity after "stopping" the reaction.

As already mentioned, arabinoxylan chains are linked to a three-dimensional network by phenolic acids ester-bound to the arabinose moieties, which form a covalent bond via their aromatic ring. During malting, these phenolic acids are released by the enzyme feruloyl esterase. Thus, in the next series of experiments, feruloyl esterase activities were determined in barley and wheat to get a deeper insight if the free phenolic acid contents can be correlated to the feruloyl esterase activities. In average, unmalted barley varieties (3.1−4.6 U/g dm) showed slightly lower activities compared to the unmalted wheat varieties (4.3−5.2 U/g dm) (Table 3). During malting, the feruloyl esterase activities in both types of grain increased. However, the increase was higher within the barley varieties with activities between 10.8 and 14.7 U/g dm compared to the wheat varieties with activities between 4.8 and 8.3 U/g dm. Since feruloyl esterase activities were significantly higher in the malted barley varieties and no clear differences in total cinnamic and ferulic acid concentrations were found between the barley and wheat varieties,[13] it was assumed that barley malts should also have higher free phenolic acid contents. However, this assumption was not reflected by the free phenolic acid contents in those malts as both types of grain had varieties with higher or lower contents of free phenolic acids compared to each other.[13] Therefore, it was suggested that the complex interaction between enzyme activities, inhibitor proteins, phenolic acid contents, etc. in the respective variety overlapped the effect of the feruloyl esterase activity.

Table 3. Feruloyl Esterase Activities in Four Barley and Three Wheat Varieties and in Malts Produced Thereof

		feruloyl esterase activity (U/g dry mass)[a]	
type	variety	grain	malt
barley	Catamaran	3.1 ± 0.1	12.9 ± 0.3
	Solist	4.2 ± 0.1	11.5 ± 0.2
	Quench	4.1 ± 0.1	10.8 ± 0.4
	Liga	4.6 ± 0.1	14.7 ± 0.7
wheat	Elixer	5.2 ± 0.1	8.0 ± 0.5
	Pannonia	4.3 ± 0.1	8.3 ± 0.4
	Torp	4.8 ± 0.1	4.8 ± 0.3

[a]Data are expressed as the mean ± standard deviation (technical: n = 3 (barley), n = 4 (wheat); analytical: n = 3).

Determination of α- and β-Amylase Activities in Grain and Malts of Barley and Wheat. α- and β-Amylase are not directly linked to the release of phenolic acids but are of general interest in the production process of beer as they are responsible for the degradation of starch, which is followed by a subsequent conversion into ethanol during fermentation. Too low activities would lead to an incomplete saccharification of starch, leading to a bad extract yield from the applied malts. Therefore, their activities are a quality parameter if the malts are also applicable for beer production. α-Amylase activities in unmalted grain of barley and wheat were negligible (0.01−0.04 U/g dm). During malting, a massive increase of the α-amylase activities between 119 and 202 U/g dm in barley malts and between 23.9 and 56.8 U/g dm in wheat malts was found (Table 4). These data were similar to the study of Sato et al.,[44] who found α-amylase activities in malts of 333 barley varieties between 21 and 460 U/g, whereby the majority of the barley varieties showed activities between 80 and 200 U/g. In the case of the wheat malts of the present study, the activities were much lower compared to the study by Burberg et al.,[45] who found α-amylase activities in malts of 13 wheat varieties between 184 and 300 U/g. However, considering the broad range of activities found in the study of Sato et al.,[44] this might also be an explanation for the wheat varieties used in this study.

In contrast to α-amylase and most other enzymes,[46] β-amylase is not expressed during malting but is formed during the development of the grain.[47] However, at that stage, its activity is controlled by the formation of disulfide bridges to an inhibitor protein.[47−49] The present β-amylase activities found in the unmalted barley and wheat varieties are, therefore, a consequence of the applied test conditions. The use of cysteine in the extraction buffer led to a reduction of the disulfide bridges, resulting in a complete release of β-amylase from the inhibitor protein. In fact, this early formation in grain of β-amylase explains why only tiny changes in the β-amylase activities were found between the unmalted and malted barley and wheat varieties (Table 4). The small decreases of the β-amylase activities (except for Pannonia) during malting can be traced back to the fact that kiln-drying is a dry heating process hardly affecting enzyme activities. A comparison to the study of Hidalgo et al.[50] reporting on the β-amylase activities in 59 different wheat varieties, with activities between 12 and 67 U/g dm, confirmed the activities found in the present study with β-amylase activities between 11.4 and 18.3 U/g dm in barley malts and between 25.3 and 40.8 U/g dm in wheat malts (Table 4).

Influence of the Protease Activity on Enzymes Directly or Indirectly Related to the Release of Phenolic Acids. During germination, the seedling does not only need sugars to grow but also needs free amino acids, which explains the expression of proteases during malting to degrade storage proteins. To what extent these proteases also have an impact on the enzyme activities of feruloyl esterase, α- and β-amylase, and β-glucanase was determined next within the current study. In unmalted grain, protease activities of barley varieties were approximately 50−75% of the activities found in wheat

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

Table 4. α- and β-Amylase Activities in Four Barley and Three Wheat Varieties and in Malts Produced Thereof

		enzyme activity (U/g dry mass)[a]			
		α-amylase		β-amylase	
type	variety	grain	malt	grain	malt
barley	Catamaran	0.010 ± 0.001	162.1 ± 3.3	16.3 ± 0.1	13.8 ± 0.1
	Solist	0.029 ± 0.003	201.9 ± 8.0	13.6 ± 0.1	11.4 ± 0.2
	Quench	0.022 ± 0.002	119.0 ± 3.1	19.0 ± 0.2	15.0 ± 0.2
	Liga	0.013 ± 0.003	136.6 ± 1.0	27.1 ± 0.1	18.3 ± 0.1
wheat	Elixer	0.030 ± 0.003	47.9 ± 0.7	43.9 ± 1.5	40.8 ± 1.2
	Pannonia	0.036 ± 0.002	56.8 ± 2.3	29.9 ± 0.3	31.1 ± 0.4
	Torp	0.031 ± 0.003	23.9 ± 1.1	35.0 ± 0.2	25.3 ± 0.4

[a]Mean values of triplicates (± standard deviation).

Table 5. Protease Activities under Non-Reducing and Reducing (with Cysteine) Conditions in Four Barley and Three Wheat Varieties and in Malts Produced Thereof

		enzyme activity (U/g dry mass)[a]			
		protease		protease with cysteine	
type	variety	grain	malt	grain	malt
barley	Catamaran	3.7 ± 0.2	25.9 ± 0.7	12.8 ± 1.2	43.8 ± 1.1
	Solist	6.1 ± 0.3	31.9 ± 0.9	15.2 ± 0.7	51.4 ± 0.7
	Quench	5.4 ± 0.6	32.7 ± 1.1	14.6 ± 0.9	42.1 ± 0.5
	Liga	2.7 ± 0.3	26.6 ± 0.3	12.0 ± 0.8	39.8 ± 0.4
wheat	Elixer	7.7 ± 0.6	28.5 ± 1.1	16.7 ± 0.7	41.8 ± 1.0
	Pannonia	8.1 ± 0.2	38.8 ± 0.4	12.7 ± 0.2	50.5 ± 1.7
	Torp	7.1 ± 0.3	35.0 ± 0.4	19.8 ± 0.6	41.0 ± 0.5

[a]Mean values of triplicates (± standard deviation).

varieties (Table 5). By application of reducing conditions, using cysteine, a strong increase of the protease activity was found in all cases, which depicts the high amount of cysteine proteases in regard to the overall activity. During malting, a massive increase of the protease activities took place from 2.7−8.1 U/g dm in unmalted barley and wheat grain to 25.9−38.8 U/g dm in barley and wheat malts under non-reducing conditions. On the one hand, the differences of the protease activities in malts between barley and wheat were less pronounced compared to both types of unmalted grain. On the other hand, reducing conditions led to a stronger increase of the protease activities in the unmalted grain compared to the malts (Table 5). Together with the dominance of the enzyme activity in grain by cysteine proteases and the most distinct increase found from grain to malt, this led to the assumption that the expression of cysteine proteases was smaller than other protease classes during malting.

Calculated Pearson correlation coefficients between the protease activity and the feruloyl esterase, α- and β-amylase, and β-glucanase activities revealed that the protease activity had no effect on the activities of these other enzymes except for α-amylase in grain under non-reducing conditions (r = 0.95, p-value < 0.001; Table 6). This can be ascribed to the task of proteases to degrade the protein matrix in which the starch granules are embedded, enabling the access of other enzymes to degrade the starch as an energy source for the seedling on the one hand and to provide the seedling free amino acids on the other hand.

In summary, the results of this study revealed that (i) during the production of malts for wheat beer brewing, phenolic acids are not trapped inside of intact cell walls as the β-glucan contents indicated a complete depletion. (ii) Concerning the impact of the solubilization of cell wall polysaccharides, higher amounts of soluble phenolic acids in wheat wort compared to barley wort could be ascribed to higher water-extractable arabinoxylan contents in the corresponding wheat malts. (iii) In contrast, no correlation was found between the feruloyl esterase activity and the free phenolic acid contents in malts, which demonstrated that the feruloyl esterase activity is not exclusively responsible for the differences in the free phenolic acid contents in the respective varieties and that additional factors contribute to these differences, e.g., inhibitor proteins and the total phenolic acid contents. (iv) Also, no correlation was found between the protease activity and the activities of further enzymes, directly or indirectly involved in the release of

Table 6. Pearson Correlation Coefficients between Protease Activities under Non-Reducing and Reducing (with Cysteine) Conditions and Feruloyl Esterase, α- and β-Amylase, and β-Glucanase Activities in Grain and Malts[a]

enzyme	feruloyl esterase	α-amylase	β-amylase	β-glucanase
	grain			
protease	0.47	0.95***[b]	0.53	0.36
protease with cysteine	0.54	0.52	0.48	0.26
	malt			
protease	−0.67	−0.48	0.25	−0.63
protease with cysteine	−0.03	0.32	−0.13	−0.11

[a]n = 7. [b]Significance at p-value < 0.001.

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

phenolic acids. With the focus on the mitigation of the undesired toxicologically relevant styrene in wheat beer via a reduction of its precursor cinnamic acid and the maintenance of still sufficient amounts of the precursors of the desired aroma-active vinyl aromatics 4-vinylphenol and 2-methoxy-4-vinylphenol, namely, *p*-coumaric acid and ferulic acid, parameters affecting enzymes involved in the degradation of arabinoxylan are the most promising tools for styrene mitigation among the effects evaluated in the present study.

AUTHOR INFORMATION

Corresponding Author

Michael Granvogl − *Institut für Lebensmittelchemie, Fachgebiet Lebensmittelchemie und Analytische Chemie (170a), Fakultät Naturwissenschaften, Universität Hohenheim, D-70599 Stuttgart, Germany;* orcid.org/0000-0003-1281-9999; Phone: +49 711 459 23979; Email: michael.granvogl@uni-hohenheim.de; Fax: +49 711 459 24096

Authors

Valerian Kalb − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany*

Torsten Seewald − *Institut für Lebensmitteltechnologie und Lebensmittelchemie, Fachgebiet Brauwesen, Technische Universität Berlin, D-13353 Berlin, Germany*

Thomas Hofmann − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany;* orcid.org/0000-0003-4057-7165

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c04837

Funding

The IGF Project (AiF 19593 N) of the Association for the Promotion of Science of the German Brewing Industry (Wifoe) was supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a decision by the German Bundestag.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Miss Anneliese Köhler for technical assistance.

REFERENCES

(1) Renner, R.; Bartels, H.; Tressl, R. Enrichment and quantitative determination of beer aroma substances by gas extraction (in German). *Monatsschr. Brau.* **1976**, *29*, 478−480.

(2) Tressl, R.; Friese, L.; Fendesack, F.; Koeppler, H. Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. *J. Agric. Food Chem.* **1978**, *26*, 1422−1426.

(3) WHO. *Guidelines for Drinking-Water Quality: Health Criteria and Other Supporting Information*, 2nd ed.; World Health Organization: Geneva, Switzerland, 1996.

(4) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicol. Sci.* **1998**, *46*, 266−281.

(5) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Bevan, C.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J. Appl. Toxicol.* **2001**, *21*, 185−198.

(6) IARC. *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, some Mycotoxins, Naphtalene and Styrene*; International Agency for Research on Cancer: Lyon, France, 2002.

(7) Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. *J. Agric. Food Chem.* **2013**, *61*, 11303−11311.

(8) Langos, D.; Granvogl, M. Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. *J. Agric. Food Chem.* **2016**, *64*, 2325−2332.

(9) Daly, B.; Collins, F.; Madigan, D.; Donelly, D.; Coakley, M.; Ross, P. An investigation into styrene in beer. *Proc. Congr. - Eur. Brew. Conv.* **1997**, *26*, 623−630.

(10) Goodey, A. R.; Tubb, R. S. Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *J. Gen. Microbiol.* **1982**, *128*, 2615−2620.

(11) Schwarz, K. J.; Boitz, L. I.; Methner, F.-J. Release of phenolic acids and amino acids during mashing dependent on temperature, pH, time and raw materials. *J. Am. Soc. Brew. Chem.* **2012**, *70*, 290−295.

(12) Langos, D.; Granvogl, M.; Gastl, M.; Schieberle, P. Influence of malt modifications on the concentrations of free phenolic acids in wheat and barley malts. *Brew. Sci.* **2015**, *68*, 93−101.

(13) Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Studies on the impact of malting and mashing on the free, soluble ester-bound, and unsoluble ester-bound forms of desired and undesired phenolic acids aiming at styrene mitigation during wheat beer brewing. *J. Agric. Food Chem.* **2020**, DOI: 10.1021/acs.jafc.0c04835.

(14) Pfenninger, H. *Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommision (MEBAK)*, 3rd ed.; Brautechnische Analysenmethoden 1; Selbstverlag der MEBAK: Freising, Germany, 1997.

(15) Andersson, A. A. M.; Lampi, A.-M.; Nyström, L.; Piironen, V.; Li, L.; Ward, J. L.; Gebruers, K.; Courtin, C. M.; Delcour, J. A.; Boros, D.; Fraś, A.; Dynkowska, W.; Rakszegi, M.; Bedő, Z.; Shewry, P. R.; Åman, P. Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.* **2008**, *56*, 9767−9776.

(16) Gebruers, K.; Dornez, E.; Boros, D.; Fraś, A.; Dynkowska, W.; Bedő, Z.; Rakszegi, M.; Delcour, J. A.; Courtin, C. M. Variation in the content of dietary fiber and components thereof in wheats in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.* **2008**, *56*, 9740−9749.

(17) Sungurtas, J.; Swanston, J. S.; Davies, H. V.; McDougall, G. J. Xylan-degrading enzymes and arabinoxylan solubilisation in barley cultivars of differing malting quality. *J. Cereal Sci.* **2004**, *39*, 273−281.

(18) Krahl, M.; Müller, S.; Back, W. Arabinoxylan (pentosane) in malt and beer production (in German). *Brauwelt* **2008**, *10*, 248−252.

(19) European Brewery Convention. *Analytica - EBC*, 9th ed.; Fachverlag Hans Carl: Nuremberg, Germany, 2010.

(20) Mastihuba, V.; Kremnický, L.; Mastihubová, M.; Willett, J. L.; Côté, G. L. A spectrophotometric assay for feruloyl esterases. *Anal. Biochem.* **2002**, *309*, 96−101.

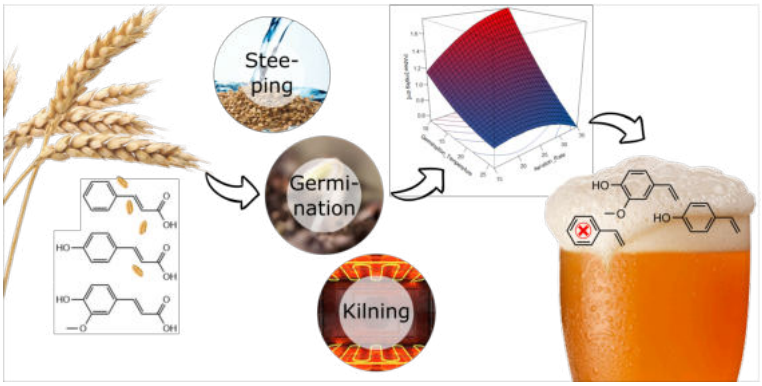
(21) Vanbeneden, N.; Gils, F.; Delvaux, F.; Delvaux, F. R. Variability in the release of free and bound hydroxycinnamic acids from diverse malted barley (*Hordeum vulgare* L.) cultivars during wort production. *J. Agric. Food Chem.* **2007**, *55*, 11002−11010.

(22) Großmann, I. Enzymatic Modification of the Arabinoxylan and Protein Fractions of Rye Dough for the Elucidation and Optimization of Structure Building Properties (in German). Ph.D. thesis, Technical University of Munich: Munich, Germany, 2017.

(23) Rani, K. U.; Prasada Rao, U. J. S.; Leelavathi, K.; Haridas Rao, P. Distribution of enzymes in wheat flour mill streams. *J. Cereal Sci.* **2001**, *34*, 233−242.
(24) Brijs, K.; Trogh, I.; Jones, B. L.; Delcour, J. A. Proteolytic enzymes in germinating rye grains. *Cereal Chem. J.* **2002**, *79*, 423−428.
(25) Jones, B. L. Endoproteases of barley and malt. *J. Cereal Sci.* **2005**, *42*, 139−156.
(26) Bell, A. Analysis of the Barley Grain Protease Spectrum. Ph.D. thesis, Heriot-Watt University: Edinburgh, Great Britain, 2012.
(27) Eason, J. R.; West, P. J.; Brummell, D. A.; Watson, L. M.; Somerfield, S. D.; McLachlan, A. R. G. Overexpression of the protease inhibitor *BoCPI-1* in broccoli delays chlorophyll loss after harvest and causes down-regulation of cysteine protease gene expression. *Postharvest Biol. Technol.* **2014**, *97*, 23−31.
(28) Singh, J.; Vohra, R. M.; Sahoo, D. K. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. *Biotechnol. Lett.* **1999**, *21*, 921−924.
(29) R Development Core Team. *A language and environment for statistical computing*; R Foundation for Statistical Computing: Vienna, Austria, 2019; http://www.r-project.org/(accessed April 14, 2020).
(30) RStudio Team. *RStudio: integrated development for R*; RStudio: Boston, MA, 2019; http://www.rstudio.com (accessed April 14, 2020).
(31) Harrell, F. E. *R Hmisc: harrell miscellanous*; 2019; https://cran.r-project.org/package=Hmisc (accessed April 14, 2020).
(32) Dvořáková, M.; Guido, L. F.; Dostálek, P.; Skulilová, Z.; Moreira, M. M.; Barros, A. A. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *J. Inst. Brew.* **2008**, *114*, 27−33.
(33) Nordkvist, E.; Salomonsson, A.-C.; Åman, P. Distribution of insoluble bound phenolic acids in barley grain. *J. Sci. Food Agric.* **1984**, *35*, 657−661.
(34) Maillard, M.-N.; Berset, C. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. *J. Agric. Food Chem.* **1995**, *43*, 1789−1793.
(35) Fincher, G. B. Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. *Plant Physiol.* **2009**, *149*, 27−37.
(36) Fincher, G. B. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 305−346.
(37) Gubler, F.; Ashford, A. E. Release of ferulic acid esters from barley aleurone. I. Time course of gibberellic-acid-induced release from isolated layers. *Aust. J. Plant Physiol.* **1985**, *12*, 297−305.
(38) Kunze, W. *Technology Brewing and Malting*, 5th English ed.; Versuchs- und Lehranstalt für Brauerei: Berlin, Germany, 2016.
(39) Adom, K. K.; Sorrells, M. E.; Liu, R. H. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *J. Agric. Food Chem.* **2005**, *53*, 2297−2306.
(40) McNeil, M.; Albersheim, P.; Taiz, L.; Jones, R. L. The structure of plant cell walls. VII. Barley aleurone cells. *Plant Physiol.* **1975**, *55*, 64−68.
(41) Hübner, F.; Arendt, E. K. Germination of cereal grains as a way to improve the nutritional value: a review. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 853−861.
(42) Debyser, W.; Delvaux, F.; Delcour, J. A. Activity of arabinoxylan hydrolyzing enzymes during mashing with barley malt or barley malt and unmalted wheat. *J. Agric. Food Chem.* **1998**, *46*, 4836−4841.
(43) Wang, D.; Meng-meng, G.; Jin-hua, D. Determination and characterisation of feruloyl esterase in wheats and malts. *Food Ferment. Ind.* **2011**, *37*, 181−185.
(44) Sato, K.; Hisano, H.; Matsumoto, S.; Zhou, T.-S.; Kihara, M. Detection of QTLs controlling alpha-amylase activity in a diversity panel of 343 barley accessions. *Mol. Breeding* **2018**, *38*, 1−10.
(45) Burberg, F.; Hartmann, K.; Zarnkow, M.; Krahl, M.; Back, W. Quality brewing wheat - results of smaller malting with the variety consideration 2007 (in German). *Brauwelt* **2008**, *36*, 1030−1034.
(46) MacGregor, A. W.; Lenoir, C. Studies on α-glucosidase in barley and malt. *J. Inst. Brew.* **1987**, *93*, 334−337.
(47) Hara-Nishimura, I.; Nishimura, M.; Daussant, J. Conversion of free β-amylase to bound β-amylase on starch granules in the barley endosperm during desiccation phase of seed development. *Protoplasma* **1986**, *134*, 149−153.
(48) Daussant, J.; Corvazier, P. Biosynthesis and modifications of α- and β-amylases in germinating whead seeds. *FEBS Lett.* **1970**, *7*, 191−194.
(49) Hejgaard, J. Free and protein-bound β-amylases of barley grain. Characterization by two-dimensional immunoelectrophoresis. *Physiol. Plant.* **1976**, *38*, 293−299.
(50) Hidalgo, A.; Brusco, M.; Plizzari, L.; Brandolini, A. Polyphenol oxidase, alpha-amylase and beta-amylase activities of *Triticum monococcum*, *Triticum turgidum* and *Triticum aestivum*: A two-year study. *J. Cereal Sci.* **2013**, *58*, 51−58.

https://dx.doi.org/10.1021/acs.jafc.0c04837
J. Agric. Food Chem. 2020, 68, 13888−13896

3.4 The Malting Parameters: Steeping, Germination, Withering, and Kilning Temperature and Aeration Rate as Possibilities for Styrene Mitigation in Wheat Beer



3.4.1 Summary

Within the scope of this study, the ability of various malting parameters for styrene mitigation should be examined in more detail. Therefore, selected parameters, including the steeping temperature, the germination temperature in combination with the aeration rate, and the temperatures applied during kiln-drying, were varied during malt preparation and their impact on the total, soluble, and free phenolic acids contents, total and water-extractable arabinoxylan contents, and feruloyl esterase activity was evaluated in the respective samples. From the malts, corresponding wort was prepared to study how changes in the malts affected the situation in wort.

Results from the various malting experiments confirmed the suitability of the selected malting parameters for styrene mitigation, and additionally, allowed to identify conditions for the reduction of the undesired precursor cinnamic acid in malts, and thus, also in wort. Aiming at the mitigation of styrene, data of the present study clearly recommended the use of high steeping temperatures, high germination temperatures combined with low aeration rates, and low withering temperatures when preparing barley malts, and medium steeping temperatures, high germination temperatures combined with low aeration rates, and low withering temperatures when preparing wheat malts.
The withering temperature during kiln-drying proved to have the strongest impact among the evaluated malting parameters, with the capacity to reduce the soluble cinnamic acid content in wort by up to 72%. Second was the germination temperature together with the aeration rate, showing a reduction capacity of up to 52%, whereby the aeration rate played only a minor role. The smallest impact, with a reduction capacity of 16% at most, was found for the steeping temperature, which could be ascribed to a temperature dependency of the enzymes involved in the release of phenolic acids, showing no great differences in the applied range. Indeed, conditions favourable for lower cinnamic acid contents also had a reducing effect on the contents

of the desired precursors. However, overall still sufficient amounts of *p*-coumaric and ferulic acid were found in the respective wort, guaranteeing the formation of the distinct aroma of wheat beer expected by the consumers.
Furthermore, the analysis of malts kilned at 80 ℃ and 200 ℃ revealed that thermal decarboxylation is only one of the factors responsible for the lower vinyl aromatic contents in dark wheat beers. In contrast, the lack of an additional release of *p*-coumaric and ferulic acid by the feruloyl esterase during mashing, an unusual low transfer of already soluble phenolic acids, and the absence of an additional biosynthesis, normally found for cinnamic acid, were found to be the main reasons for the low phenolic acid contents in dark wort. Since in malts kilned at 200 ℃ no feruloyl esterase activity was present anymore, thus explaining the absent release of further phenolic acids during mashing, it was assumed that also other enzymes, involved in the degradation of arabinoxylan and the biosynthesis of phenolic acids, were affected by the high kilning temperatures applied during the preparation of dark malts.
Finally, to evaluate the applicability of these potential new styrene mitigation strategies in wheat beer brewing, malts were analyzed if they fulfil certain specifications (moisture content, extract, colour, pH-value, total protein content, Kolbach index, FAN (free amino nitrogen), and soluble β-glucan content [81, 82]). The high agreement with the standard quality values for malt and the values of commercial malts confirmed the applicability of the prepared malts for wheat beer brewing, thus underlining the importance of malting as a tool for styrene mitigation.

The individual contributions for achievement of V. Kalb and the co-authors' contributions are specified below:

V. Kalb developed the design of the study and conducted the laboratory work. Malt and wort preparation and malt analysis were performed by T. Seewald at the Chair of Brewing Science and Beverage Technology, Technische Universität Berlin. A. Köhler, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, assisted in sample preparation and S. Kaviani-Nejad, Leibniz-Institute for Food System Biology at the Technical University of Munich, was involved in the mass spectrometric determination of phenolic acids via HPLC-MS/MS. V. Kalb statistically analysed and interpreted the data, discussed the results, and created the figures and tables. He developed the story of the paper, wrote the first draft of the manuscript, and revised the paper. T. Hofmann, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, coordinated the research project. Prof. Dr. M. Granvogl, Institute of Food Chemistry, University of Hohenheim, was responsible for the conceptualization, project administration, supervision of the research project, and writing reviews and editing.

3.4.2 Publication

The Malting Parameters: Steeping, Germination, Withering, and Kilning Temperature and Aeration Rate as Possibilities for Styrene Mitigation in Wheat Beer

V. Kalb, T. Seewald, T. Hofmann, and M. Granvogl

Permanent weblink: https://dx.doi.org/10.1007/s00217-021-03852-5

European Food Research and Technology
https://doi.org/10.1007/s00217-021-03852-5

ORIGINAL PAPER

The malting parameters: steeping, germination, withering, and kilning temperature and aeration rate as possibilities for styrene mitigation in wheat beer

Valerian Kalb[1] · Torsten Seewald[2] · Thomas Hofmann[1,3] · Michael Granvogl[4]

Received: 16 April 2021 / Revised: 20 August 2021 / Accepted: 27 August 2021

Abstract
Aiming at the mitigation of the toxicologically relevant styrene formed during wheat beer brewing, different malting parameters, such as steeping temperature, germination temperature, withering and kilning temperatures applied during kiln-drying, and aeration rate, were evaluated for their suitability to reduce the content of cinnamic acid, the precursor of styrene, in malts of barley and wheat, responsible for the input of the undesired precursor into the brewing process. According to the results of the present study, higher steeping temperatures, higher germination temperatures, lower aeration rates, and lower withering temperatures during malting are beneficial for the overall reduction of cinnamic acid in wort produced with barley and wheat malts. Thereby, the withering temperature showed the highest impact among the investigated parameters, able to reduce the soluble cinnamic acid content in wort by up to 72%, followed by the germination temperature in combination with the aeration rate and the steeping temperature with reduction capacities of 52 and 16%, respectively. Additionally, a kilning temperature of 200 °C led to the absence of enzyme activities in dark malts, which might also be the main reason for the low phenolic acid contents found in the corresponding wort, finally causing the low concentrations of styrene but also to a certain extent of desired vinyl aromatics in dark wheat beers.

Keywords Wheat beer · Phenolic acids · Malting · Steeping · Germination · Aeration rate · Kiln-drying

Introduction

Toxicologically relevant compounds in groceries are not always an issue of contamination, e.g., from environmental pollution, cultivation, or packaging, but they can also be present as natural occurring components or formed during food processing [1–3]. This is also the case for wheat beer, a special beer type originating from the southern part of Germany (Bavaria), which contains styrene in relevant amounts. The presence of styrene in wheat beer is known since the late 1970s [4, 5]; however, its toxicological relevance was not considered until 1996, when a tolerable daily intake (TDI) of 7.7 µg/kg body weight per day was published by the World Health Organization (WHO) [6]. This first toxicological assessment was then updated in 2002 by the International Agency for Research on Cancer (IARC), which classified styrene as possibly carcinogenic to humans (class 2B) [7], based on the results of studies on rats [8] and mice [9]. According to different studies [10–12], styrene concentrations can be expected within the range of 9.8–33 µg/L, not exceeding the established TDI by normal wheat beer

✉ Michael Granvogl
michael.granvogl@uni-hohenheim.de

1 Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, 85354 Freising, Germany

2 Institut für Lebensmitteltechnologie und Lebensmittelchemie, Fachgebiet Brauwesen, Technische Universität Berlin, 13353 Berlin, Germany

3 Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München, Lise-Meitner-Straße 34, 85354 Freising, Germany

4 Fachgebiet Lebensmittelchemie und Analytische Chemie (170a), Institut für Lebensmittelchemie, Fakultät Naturwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany

Published online: 19 October 2021

consumption. Nevertheless, mitigation strategies should be applied also to wheat beer to increase consumer's safety.

The difficulty of this objective is given by similar formation pathways of styrene and two key aroma compounds in wheat beer, namely 4-vinylphenol and 2-methoxy-4-vinylphenol, which are mainly responsible for the formation of the well-known and characteristic clove-like and slightly phenolic aroma of wheat beer [13]. These three compounds derive from the precursors cinnamic acid, *p*-coumaric acid, and ferulic acid, which belong to the same compound class of cinnamic acid derivatives, also known as phenolic acids. The major sources of phenolic acids in the brewing process are barley and wheat malts. By law, the content of wheat malt has to be at least 50%. In both barley and wheat, phenolic acids fulfill multiple tasks. Based on the phenylpropanoid metabolism, they are the major building blocks for the formation of flavonoids, stilbenoids, and lignin [14, 15]. Additionally, they are able to link the linear polysaccharide chains of arabinoxylan to a three-dimensional network [16–18], which build up the cell walls in grain of barley and wheat. This structure is seen as the main source of precursors of the desired and undesired vinyl aromatics in wheat beer [19–21].

Within the first steps (malting and mashing) of the brewing process, different enzymes lead to the degradation of the polysaccharide backbone of the arabinoxylan structure [22]. Simultaneously, the feruloyl esterase directly splits off the phenolic acids from arabinoxylan. As a consequence, three different phenolic acid forms are formed according to the size of the fragments, the insoluble ester-bound phenolic acids, the soluble ester-bound phenolic acids, and the free phenolic acids. However, only the soluble forms (the soluble ester-bound and free phenolic acids) can be transferred from malt into wort during mashing and are, therefore, later on accessible by yeasts. In the following fermentation step, the free phenolic acids are decarboxylated to the corresponding desired and undesired vinyl aromatics by yeasts. Their decarboxylation ability is related to an active POF1 gene. A study of Daly et al. [12] revealed that bottom-fermenting yeasts lack this ability without any exception, whereas top-fermenting yeasts have either no, a reduced, or a high Pof-activity. Typically, wheat beers are brewed with top-fermenting yeasts with high Pof-activities, leading to the formation of styrene in relevant amounts. Consequently, styrene does not play a significant role in beers brewed with a bottom-fermenting yeast, e.g., lager beers, or in wheat beers brewed with a top-fermenting yeast having no/strongly reduced Pof-activity [13].

Several studies on the reduction of styrene in wheat beer were examined by Langos and Granvogl [11], Langos et al. [23, 24], Schwarz and Methner [25], and Schwarz et al. [26–29], mainly focusing on the impact of brewing steps after malting, such as mashing, wort boiling, and fermentation. Thereby, found styrene mitigation strategies within these processing steps were mostly accompanied by a significant reduction of the desired vinyl aromatics and, therefore, with the risk to lose the characteristic wheat beer aroma. Thus, another mitigation approach of styrene aimed at the reduction of its precursor cinnamic acid by altering the malting parameters. In a first study performed by Langos et al. [24], "undermodified" malts (malting parameters: steeping degree, 42%; germination temperature, 12 °C; germination time, 5 days) showed a clear reduction of the undesired cinnamic acid (56% for barley, 30% for wheat) in comparison to "highly modified" malts (malting parameters: steeping degree, 48%; germination temperature, 18 °C; germination time, 7 days), proving that the malting process is a promising tool to lower the styrene content in wheat beer.

The aim of the present study was to get a better understanding of the impact of various malting parameters on the release of the different forms of phenolic acids and the enzymatic degradation of the arabinoxylan structure. Further, this knowledge should be a toolbox for brewers to establish beneficial malting conditions allowing a reduction of the styrene content in wheat beer. Therefore, barley and wheat malts were produced by either varying (1) the steeping temperature, (2) the germination temperature in combination with the aeration rate, or (3) the withering temperature and (4) the kilning temperature both applied during kiln-drying. Within these malts and congress wort produced thereof, the insoluble ester-bound, soluble ester-bound, and the free cinnamic, *p*-coumaric, and ferulic acid contents were determined, as well as the amount of water-extractable arabinoxylan and the feruloyl esterase activity.

Materials and methods

Malt samples and congress wort

Malting and mashing experiments as well as the standard brewing analysis according to MEBAK® [30] and EBC [31] were performed by the Chair of Brewing Science and Beverage Technology (Technical University of Berlin, Berlin, Germany). The sample set included two industrially relevant barley varieties, namely, Solist (spring barley (sp), two-rowed (2r), Franconia, Germany) and Quench (sp, 2r, Denmark), both harvest year 2017, and one industrially relevant wheat variety, namely, Dacanto (winter wheat, Denmark), harvest year 2016. The standard malting conditions selected within this study were closely related to the parameters normally used by malthouses: a steeping degree of 45% was adjusted by steeping the grain in a physiological saline solution (0.9%) for 4 h at 16 °C, followed by an air rest for 20 h at a flow rate of 25 L/min and an air humidity of 80%. This cycle was

repeated with an additional steeping for 3 h and an air rest for 21 h. Next, the grain was germinated for 5 days at 18 °C. The green malts were withered at a temperature of 50 °C for 16 h, followed by kilning at a temperature of 80 °C for 4 h to reach a water content < 5%. Subsequently, the kilned malts were deculmed. These standard malting conditions have been adapted to different experiments to study the impact of the malting parameters on the formation and release of the desired and undesired precursors of the vinyl aromatics during malt production. In experiment **1,** the steeping temperature was varied between 10, 20, and 30 °C, covering a broad temperature range allowing the visualization of the impact of this malting parameter on the target analytes. For experiment **2,** the response surface methodology (RSM) was employed to investigate the variation of the analytes of interest with respect to the temperature in combination with the aeration rate during germination. The composition of the two variables was designed by the central composite design approach, which is a 2^k factorial design with $2k$ star points and central points. With $\alpha = \pm 1$ as the relative distance from the center of the design to a factorial point, the relative distance of a star point is $\alpha = 2^{k/4}$. The ranges of the variables were defined as follows: germination temperature, 10–26 °C; aeration rate, 15–35 L/min. For reference purposes, malts were balanced according to their malting degree. Therefore, the germination time was extended to 6 days for malts with a germination temperature of 10 and 12 °C. In total, 14 experimental settings consisting of 4 factorial, 4 star, and 6 central points were generated on the basis of the two variables of the experiment (Table 1). Within experiment **3,** the withering temperature was varied between 30, 40, 50, and 60 °C and maintained for 16 h. For the production of pale malts, the kilning temperature was set to 80 °C for 4 h to reach a water content < 5%. Again, the kilned malts were directly deculmed. To study the impact of the kilning temperature within experiment **4,** a set of dark malts was additionally produced to the pale malts with a withering temperature of 50 °C and a kilning temperature of 200 °C (2 min). Further, from all these malts, the corresponding wort was produced according to the standard mashing procedure of congress mash (50 g of malt, 400 mL of water) (MEBAK® 4.1.4.2 [30], EBC 4.5.1 [31]), since a former study [35] revealed that changes within the malts can differ from the impact that varying malting parameters have on the final state in wort.

Table 1 Coded variables and the corresponding process variables of the central composite design for the evaluation of the impact of the germination temperature in combination with the aeration rate on the release of the different forms of phenolic acids and the enzymatic degradation of the arabinoxylan structure during malting

Experimental setting	Coded variables		Process variables	
	X_1[a]	X_2[b]	X_1[a]	X_2[b]
1	− 1	− 1	12	18
2	1	− 1	24	18
3	− 1	1	12	32
4	1	1	24	32
5	$-\sqrt{2}$	0	10	25
6	$\sqrt{2}$	0	26	25
7	0	$-\sqrt{2}$	18	15
8	0	$\sqrt{2}$	18	35
9	0	0	18	25
10	0	0	18	25
11	0	0	18	25
12	0	0	18	25
13	0	0	18	25
14	0	0	18	25

[a]Germination temperature (°C)

[b]Aeration rate (L/min)

Chemicals

The following compounds were commercially available: 2-morpholinoethanesulfonic acid (Mes) and polyethylene glycol mono(*p*-(1,1,3,3-tetramethylbutyl)phenyl) ether (Triton X-100) (AppliChem, Darmstadt, Germany); acetonitrile and methanol (Baker, Gliwice, Poland); 4-nitrophenyl *trans*-ferulate (Carbosynth, Compton, Berkshire, United Kingdom); 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris base) and *p*-nitrophenol (Carl Roth, Karlsruhe, Germany); hydrochloric acid, dimethyl sulfoxide (DMSO), formic acid (FA), and potassium dihydrogen phosphate (Merck, Darmstadt); and cinnamic acid, *p*-coumaric acid, ferulic acid, and sodium hydroxide (Sigma-Aldrich; Merck). All solvents were of HPLC gradient grade and ultrapure water was from an in-house source.

Stable isotopically labeled internal standards

The following stable isotopically labeled internal standards were commercially available: [$^2H_7$]-cinnamic acid (98 atom % ^2H), [$^{13}C_3$]-*p*-coumaric acid (99 atom % ^{13}C), and [$^{13}C_3$]-ferulic acid (99 atom % ^{13}C) (Sigma-Aldrich; Merck).

Sample pre-treatment

Prior to use, malt samples (frozen at − 18 °C) were first ground by means of a centrifugal mill (12,000 rpm, sieve opening 0.5 mm; ZM 200; Retsch, Haan, Germany) and then stored in wide-necked brown glass bottles for light exclusion at room temperature (RT).

Total and water-extractable arabinoxylan contents in malt

Total and water-extractable arabinoxylan contents in malt were determined by the application of a D-xylose assay kit (K-XYLOSE 04/16; Megazyme, Wicklow, Ireland) as previously described [32].

Briefly, total arabinoxylan contents were determined from ground samples directly hydrolyzed with hydrochloric acid (1.3 mol/L; 1 h at 100 °C; 600 rpm; HCL HeizThermoMixer MHL23; Digital Biomedical Imaging Systems, Pforzheim, Germany). The hydrolysates were neutralized with sodium hydroxide (1.3 mol/L) and adjusted to 100 mL with distilled water. Subsequently, aliquots (15 mL) were centrifuged (4600 rpm, 10 min, RT; Heraeus Multifuge X3 FR; Thermo Fisher Scientific, Dreieich, Germany) and aliquots of the supernatants (500 µL) were used within the enzyme assay.

For the determination of the water-extractable arabinoxylan content, a modified sample preparation was used. Briefly, malt samples were mixed with hot water (85 °C) followed by an incubation for 15 min at 90 °C and additional 30 min at 60 °C (600 rpm; HCL HeizThermoMixer MHL23). Then, the extracts were cooled to RT, centrifuged (4600 rpm, 10 min, RT; Heraeus Multifuge X3 FR), and the supernatants were hydrolyzed by the addition of hydrochloric acid (32%; 1 h at 100 °C). The hydrolysates were neutralized with sodium hydroxide (1.3 mol/L) and adjusted to 20 mL with distilled water. Prior to the enzyme assay, the precipitates were removed by an additional centrifugation step (10,000 rpm, 5 min, RT; Himac 15RE; VWR, Ismaning, Germany). Aliquots of the supernatants (375 µL) were then transferred together with distilled water, a buffer solution (pH value of 7.5), an NAD^+/ATP solution, and a hexokinase suspension into semi-micro cuvettes (poly(methyl methacrylate), PMMA); 1.6 mL, 1 cm light path; Ratiolab, Dreieich). After mixing and incubation for 5 min, the absorbance was measured via an UV/VIS-spectrophotometer (UV-2401 PC; Shimadzu, Duisburg, Germany) at 340 nm (A1). Further, to the same mixtures, a xylose mutarotase/β-xylose dehydrogenase solution was added, and after mixing and incubation for another 6 min, the absorbance was measured for a second time (A2). By treating a blank sample with distilled water in the same way (B1, B2), the D-xylose content was calculated from the difference of the sample absorption (A2 − A1) and the difference of the blank absorption (B2 − B1) via the Lambert-Beer law ($\varepsilon = 6300$ L/(mol*cm)). From the D-xylose content, the water-extractable arabinoxylan content was calculated considering the xylose to arabinose ratio in arabinoxylan (0.62 [33, 34]) and the condensation factor (0.88). For more details, see [32].

Feruloyl esterase activity in malt

For the determination of the feruloyl esterase activity in malts, the recently published enzyme assay was applied [32]. Briefly, feruloyl esterase was extracted from malt samples with Mes-buffer (50 mmol/L Mes, pH value of 6.0) for 30 min at RT (900 rpm; HLC HeizThermoMixer MHL23). After centrifugation (4700 rpm, 15 min, RT; Heraeus Multifuge X3 FR) and membrane filtration (0.45 µm, Minisart RC, hydrophilic, 15 mm; Sartorius, Göttingen, Germany) of the extracts, the supernatants were further used in the enzymatic assay. Therefore, aliquots of the supernatants (0.3 mL) together with Mes-buffer (0.9 mL) were pre-incubated for 5 min at 30 °C, before the 4-nitrophenyl *trans*-ferulate substrate solution (1 mmol/L in phosphate buffer (0.1 mol/L, 2.5% Triton X-100 (v/v), pH value of 6.0)) was added to start the enzymatic reaction. After exactly 2 h, the enzymatic reaction was stopped by the addition of a Tris base solution (1% Tris base (w/v), pH value of 8.5), and the absorption was measured at 400 nm (UV-2401 PC). According to the minor enzymatic activity left under these conditions, a blank of the same composition (aside from the sample extract) was included for each sample triplicate. By a precise simulation of the time between stopping of the enzymatic reaction and the measurement of the absorption, the increase in absorption due to the insufficient deactivation of the feruloyl esterase can be compensated. After subtraction of the blank absorbtion from the sample absorbtion, the feruloyl esterase activity was calculated using a calibration line of 4-nitrophenol. One unit of feruloyl esterase activity is defined as the amount of enzyme that is needed to release 1 nmol of 4-nitrophenol from 4-nitrophenyl *trans*-ferulate in 1 min under the defined assay conditions. For more details, see [32].

Phenolic acid extraction from malt and quantitation by stable isotope dilution analysis

Total, soluble, and free phenolic acids were isolated and their contents in malts were determined as previously described in detail [35].

Briefly, total phenolic acids (PAtot; = sum of free, soluble ester-bound, and insoluble ester-bound phenolic acids) in malts were determined after alkaline hydrolysis. Therefore, sodium hydroxide (2 mol/L) and the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (amounts depending on the concentrations of the analytes determined in preliminary experiments) were added to the malts, and after covering with argon, the samples were hydrolyzed for 2 h at RT (Multi-Tube Vortexer; VWR). Subsequently, the hydrolysates were neutralized with hydrochloric acid (2 mol/L), centrifuged (4700 rpm, 10 min, RT; Heraeus

Multifuge X3 FR), and membrane filtered (0.45 µm) prior to the quantitation of the phenolic acids by means of high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS).

Soluble phenolic acids (PAsol; = sum of free and soluble ester-bound phenolic acids) in malts were quantitated after a hot water extraction in combination with an alkaline hydrolysis. Therefore, malts were suspended in hot water (95 °C), followed by an incubation for 1 h at 100 °C (600 rpm; HLC HeizThermoMixer MHL23). The extracts were then cooled to RT and the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (amounts depending on the concentrations of the analytes determined in preliminary experiments) were added. After equilibration by thorough mixing (GFL Orbital Shaker 3005; Gesellschaft für Labortechnik, Burgwedel, Germany), the extracts were centrifuged twice (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR; 15,000 rpm, 5 min, RT; Himac CT 15 RE), and aliquots of the supernatants (0.5 mL) were hydrolyzed with sodium hydroxide (4 mol/L) for 2 h at RT. Then, the hydrolysates were neutralized by adding hydrochloric acid (4 mol/L). After membrane filtration (0.45 µm), the contents of phenolic acids were determined by HPLC–MS/MS.

Free phenolic acids (PAfree) in malts were directly accessible after liquid extraction. Therefore, malts, the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (amounts depending on the concentrations of the analytes determined in preliminary experiments), and the extraction solvent (methanol/water (60/40, v/v)) were transferred into Precellys tubes (15 mL; CK28L; Bertin Technologies, Montigny-le-Bretonneux, France) and stored at − 18 °C for 12 h. In the next step, the samples were homogenized by means of a Precellys bead beater (6000 rpm, 3 × 45 s, break 25 s, nitrogen cooling; Precellys Evolution; Bertin Technologies), followed by a 1 h rest at RT for equilibration. Prior to analysis by HPLC–MS/MS, the extracts were centrifuged (4700 rpm, 10 min, RT; Heraeus Multifuge X3 FR) and the supernatants were membrane filtered (0.45 µm).

Quantitation of phenolic acids in wort by stable isotope dilution analysis

The determination of soluble and free phenolic acids in wort was performed as recently described [35].

Briefly, soluble phenolic acids were accessible after alkaline hydrolysis of wort. Therefore, the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (amounts depending on the concentrations of the analytes determined in preliminary experiments) were added to aliquots (0.5 mL) of the wort. Subsequently, sodium hydroxide (4 mol/L) was added for hydrolysis (600 rpm, 2 h, RT; HCL HeizThermoMixer MHL23). After incubation, the hydrolysates were neutralized with hydrochloric acid (4 mol/L), membrane filtered (0.45 µm), and analyzed by HPLC–MS/MS.

Free phenolic acids in wort were determined after inhibition of the enzyme activities by the addition of methanol in a ratio of 1 + 1 (v + v). Next, the stable isotopically labeled internal standards [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid (amounts depending on the concentrations of the analytes determined in preliminary experiments) were added, and after equilibration by thorough mixing (GFL Orbital Shaker 3005) and membrane filtration (0.45 µm), the contents of free phenolic acids were analyzed by HPLC–MS/MS.

Quantitation of phenolic acids by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS)

Phenolic acids from the different sample preparation procedures were analyzed by HPLC–MS/MS using an UltiMate 3000 HPLC system (Thermo Scientific; Dionex Softran, Germering, Germany) equipped with a Kinetex C18 column (100 × 2.1 mm, 2.6 µm, 100 Å; Phenomenex, Aschaffenburg, Germany; column temperature, 24 °C) and connected to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific, Bremen, Germany). The injection volume was 10–20 µL and the mobile phases were 0.1% FA in water (v/v) (A) and 0.1% FA in acetonitrile (v/v) (B). The following linear gradient was applied: 0–2 min, 10% B; 2–15 min, from 10 to 90% B; 15–18 min, 90% B; 18–19 min, from 90 to 10% B; and 19–30 min, 10% B, with a flow gradient of 0.05 mL/min (0–1 min), from 0.05 to 0.2 mL/min (1–2 min), and 0.2 mL/min (2–30 min). Positive atmospheric pressure chemical ionization ($APCI^+$) was applied using the following conditions: vaporizer temperature, 250 °C; discharge current, 4.0 µA; sheath gas pressure, 30 arbitrary units (au); auxiliary gas pressure, 10 au; declustering voltage, -10 V; and capillary temperature, 300 °C. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode, and the most intensive fragment ion was chosen as the quantifier ion, together with two qualifier ions [35].

Calibration lines were determined from mixtures of known contents of the unlabeled analyte and the corresponding stable isotopically labeled internal standard in seven different concentration ratios (10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10) with good linearity ($R^2 > 0.99$) for all analytes in the applied range [35].

Parameters describing the malt quality

Moisture content, extract, color, pH value, total protein content, Kolbach index, FAN, and soluble β-glucane content of the malt samples were determined according to MEBAK® [30] and EBC methods [31].

Statistical analysis

Means and standard deviations were calculated by Microsoft Office Excel (Microsoft Corporation, Seattle, WA). Statistical analyses were performed by the R statistical software (version 3.6.0) [36] using the interface R Studio (version 1.1.423) [37]. Significant differences across groups were calculated with either ANOVA (aov), Welch test (oneway.test), or the Kruskal-Wallis test (kruskal.test) from the stats package (version 3.6.0) [36]. To evaluate which groups differ from each other, the post hoc tests pairwise *t*-test (pairwise.*t*.test, p.adjustment = holm, pool.sd = TRUE(homogen variance)/FALSE(inhomogen variance)) and Dunn test (dunnTest, method = holm) were applied from the stats and FSA packages (version 0.28.27) [38]. Considering the α inflation with multiple testing, the *p*-values of the post hoc tests were adjusted according to the method of Holm. To decide in each case of the different dataset which of these tests fits best, normal distribution and homogeneity of variances were determined using the Shapiro-Wilk test (shapiro.test) and the Levene test (leveneTest, center = median) from the packages stats and car (version 3.0.6) [39]. Further, response surface models were assessed by applying the rsm function from the rsm package (version 2.10) [40], and three-dimensional plots were generated with the persp function from the package plot3D (version 1.3) [41].

Results and discussion

Impact of the steeping temperature on the release of phenolic acids during malting

Malts of the two barley varieties Solist and Quench and the wheat variety Dacanto were produced at three different steeping temperatures of 10, 20, and 30 °C to study the suitability of this malting parameter for styrene mitigation via a reduction of its precursor cinnamic acid in malts. Figure 1 (for detailed values and statistics, see Tables S1–S3 in the Online Resources) summarizes the total, soluble, and free phenolic acid contents in these malts and wort produced thereof. Malts of the two barley varieties revealed that cinnamic, but also *p*-coumaric and ferulic acid contents tended to decrease with increasing steeping temperatures. For example, free cinnamic, *p*-coumaric, and ferulic acid contents in malts of Solist decreased by 2% from 0.97 to 0.95 mg/kg dm, by 8% from 1.42 to 1.30 mg/kg dm, and by 6% from 4.27 to 4.03 mg/kg dm, with an increasing steeping temperature. For Quench, this decrease was even more pronounced with 31% from 0.72 to 0.50 mg/kg dm, 19% from 1.08 to 0.87 mg/kg dm, and 15% from 4.12 to 3.52 mg/kg dm for free cinnamic, *p*-coumaric, and ferulic acid. These results also indicated that the impact of the steeping temperature seems to be strongest for cinnamic acid and weaker for *p*-coumaric and ferulic acid (except for cinnamic acid in Solist). Additionally, the steeping temperature seems to have a slightly higher impact on the free form of the phenolic acids and to a lesser degree on the total and soluble form. After mashing, again both soluble and free cinnamic acid contents tended to decrease with an increasing steeping temperature in wort

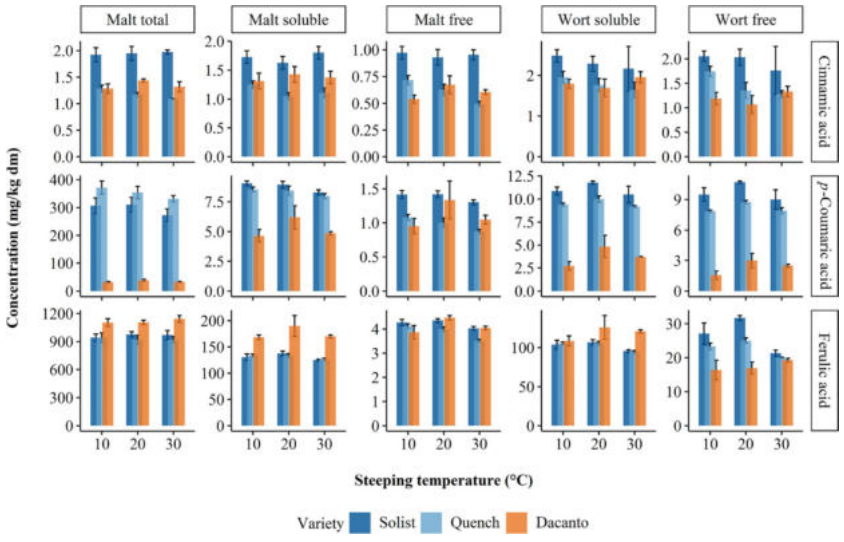


Fig. 1 Total (= sum of free, soluble ester-bound, and insoluble ester-bound), soluble (= sum of free and soluble ester-bound), and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid in dependency of the steeping temperature (10, 20, and 30 °C) in malts of the barley varieties Solist and Quench and the wheat variety Dacanto as well as in the corresponding wort (50 g of malt, 400 mL of water) produced thereof

produced from the corresponding malts. For Solist, a reduction of soluble cinnamic acid in wort of 13% and for Quench of 16% was possible using a steeping temperature of 30 °C instead of 10 °C. Interestingly, this tendency was not found for *p*-coumaric and ferulic acid for which the soluble and free contents showed the highest amounts at 20 °C (Fig. 1). Thus, for the barley varieties, a higher steeping temperature seems to be recommendable to reduce styrene via its precursor during wheat beer brewing, despite the fact that only two varieties were used in the present study. Luckily, this would also have no severe impact onto the desired aroma of wheat beer, as *p*-coumaric and ferulic acid, precursors of the desired vinyl aromatics 4-vinylphenol and 2-methoxy-4-vinylphenol, were still present at sufficient amounts.

Different from the barley varieties, total, soluble, and free phenolic acids in malts of the wheat variety Dacanto showed a concentration optimum at 20 °C. For example, in the case of the free phenolic acid contents, cinnamic acid was found with 0.67 mg/kg dm at 20 °C compared to 0.54 and 0.61 mg/kg dm (20 and 10% lower contents) at 10 and 30 °C, *p*-coumaric acid with 1.33 mg/kg dm compared to 0.95 and 1.05 mg/kg dm (28 and 21% lower contents), and ferulic acid with 4.46 mg/kg dm compared to 3.86 and 4.05 mg/kg dm (13 and 9% lower contents) (Fig. 1). After mashing, still the highest amounts of soluble *p*-coumaric and ferulic acid were found in wort produced of malts steepened at 20 °C, which was different for cinnamic acid, showing the lowest contents at this temperature. In case of soluble cinnamic acid, the content was 6 and 14% lower compared to the contents found at 10 and 30 °C. Based on these results, a steeping temperature of 20 °C seems favorable for the production of wheat malts from the perspective of styrene mitigation. Further, with a parallel increase of the desired precursors, also the maintenance of the typical wheat beer aroma expected by the consumers would be guaranteed.

In accordance with a former study [32], feruloyl esterase activities (Fig. 2; for detailed values and statistics, see Tables S1–S3 in the Online Resources), enabling the direct release of phenolic acids from arabinoxylan chains, did not explain the pattern found for the free phenolic acid contents in these malts produced at different steeping temperatures. Instead of showing a decrease with increasing steeping temperatures in the barley varieties, the feruloyl esterase activity showed an optimum for Solist and a minimum for Quench at 20 °C. Also for the wheat variety Dacanto, a different pattern compared to the free phenolic acid contents was found with the lowest feruloyl esterase activity at 20 °C. In contrast, the same study [32] confirmed a link between the degradation degree of arabinoxylan and the content of soluble phenolic acids. Also in the present study, water-extractable arabinoxylan contents (Fig. 2; for detailed values and statistics, see Tables S1–S3 in the Online Resources) showed the same pattern in dependence of the steeping temperatures as found for the soluble and free phenolic acids in malts of the barley and wheat varieties (Fig. 1). Thus, also the water-extractable arabinoxylan contents decreased with increasing steeping temperatures in the barley malts from 0.61 to 0.56 g/100 g dm (8%; Solist) and from 0.68 to 0.58 g/100 g dm (15%; Quench). In case of the wheat variety Dacanto, also an optimum was formed, with the highest water-extractable arabinoxylan content at a steeping temperature of 20 °C, revealing a content of 1.56 g/100 g dm compared to 1.36 and 1.43 g/100 g dm (13 and 8% lower contents) at 10 and 30 °C (Fig. 2). Regarding the total arabinoxylan contents, no changes were found with respect to the different steeping temperatures applied (Fig. 2). This was in accordance with the results of a previous study performed by Kalb et al. [32], in which also no significant differences were found in the total arabinoxylan contents upon malting of four barley and three wheat varieties. As an explanation for the absence of increasing amounts, the authors quoted that cell walls have to be degraded during germination to make starch and proteins accessible for the seedling, rather than building up new cell walls.

According to the results of the present study, the potential of the steeping temperature to reduce the soluble cinnamic

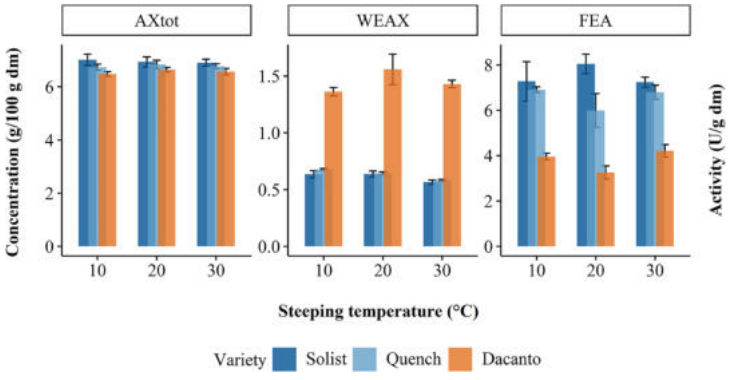


Fig. 2 Total (AXtot) and water-extractable arabinoxylan (WEAX) contents as well as feruloyl esterase activity (FEA) in dependency of the steeping temperature (10, 20, and 30 °C) in malts of the barley varieties Solist and Quench and the wheat variety Dacanto

acid content in wort within the applied temperature range can reach up to 16%. Further, the data also demonstrated that under these beneficial conditions, still sufficient amounts of the desired precursors can be expected, allowing the formation of the typical wheat beer aroma. Although clear trends were found related to the steeping temperature, statistics (Tables S1–S3 in the Online Resources) and an expected reduction of soluble cinnamic acid in wort of < 16% proved the rather small impact of the steeping temperature. However, this finding is not surprising as the temperature dependency of the feruloyl esterase [42] and of enzymes responsible for the degradation of arabinoxylan, such as *β*-D-xylosidase, *endo*-1,4-*β*-xylanase, and *α*-L-arabinofuranosidase [22], show only tiny changes in their enzyme activities in the applied temperature range. Nevertheless, the steeping temperature is one parameter which can be modified to reduce the overall styrene content in wheat beer.

Impact of the germination temperature in combination with the aeration rate on the release of phenolic acids during malting

The response surface methodology (RSM) was applied to simultaneously study the impact of the germination temperature and the aeration rate on the release of phenolic acids during malting. Therefore, 14 different malts including 4 factorial, 4 star, and 6 central points were prepared at germination temperatures ranging from 10 to 26 °C and aeration rates ranging from 15 to 35 L/min. For models with a coefficient of multiple determination (MR^2) ≥ 0.8 and a corresponding *p*-value < 0.05, the impact on the analytes of interest was considered as statistically relevant (Table 2; for each response surface plot, see Figures S1–S6 in the Online Resources). For the barley varieties Solist and Quench, it was proven that the germination temperature in combination with the aeration rate had an impact on the water-extractable arabinoxylan contents (Table 2). While increasing germination temperatures led to a decrease of the contents, increasing aeration rates had an increasing effect (Fig. 3). Thereby, the impact of the germination temperature was stronger than the influence of the aeration rate. According to the data of the present study, a reduction of the water-extractable arabinoxylan content of approximately 35% has to be expected, when using sets of parameters favorable for low water-extractable arabinoxylan contents compared to sets favorable for high contents (Fig. 3). In case of the wheat variety Dacanto, statistics showed that the water-extractable arabinoxylan content was not affected by the applied parameters. Additionally, the statistical data also showed no impact on the feruloyl esterase activity independent of the barley and wheat varieties (Table 2).

Table 2 Summary of the RSM model statistics of the impact of the germination temperature in combination with the aeration rate on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the water-extractable arabinoxylan (WEAX) content and the feruloyl esterase activity (FEA) in malts and the corresponding wort of the barley varieties Solist and Quench and the wheat variety Dacanto

Type	Phenolic acid	Solist		Quench		Dacanto	
		MR^2	*p*-value	MR^2	*p*-value	MR^2	*p*-value
Malt							
Total	Cinnamic acid	0.65	0.08	0.45	0.35	0.58	0.15
	p-Coumaric acid	0.41	0.43	0.39	0.47	0.54	0.21
	Ferulic acid	0.68	0.06	0.68	0.06	0.57	0.16
Soluble	Cinnamic acid	0.92	0.00[a]	0.73	0.03	0.57	0.16
	p-Coumaric acid	0.74	0.03	0.62	0.11	0.21	0.83
	Ferulic acid	0.30	0.65	0.43	0.39	0.56	0.18
Free	Cinnamic acid	0.85	< 0.01[a]	0.59	0.15	0.07	0.04
	p-Coumaric acid	0.80	0.01[a]	0.76	0.02	0.67	0.06
	Ferulic acid	0.93	< 0.01[a]	0.92	< 0.01[a]	0.80	0.01[a]
Wort[b]							
Soluble	Cinnamic acid	0.85	< 0.01[a]	0.42	0.41	0.86	< 0.01[a]
	p-Coumaric acid	0.47	0.31	0.35	0.54	0.31	0.63
	Ferulic acid	0.79	0.01	0.81	0.01[a]	0.72	0.04
Free	Cinnamic acid	0.82	0.01[a]	0.46	0.33	0.71	0.04
	p-Coumaric acid	0.39	0.46	0.22	0.80	0.64	0.09
	Ferulic acid	0.87	< 0.01[a]	0.14	0.92	0.64	0.09
WEAX		0.95	< 0.01[a]	0.99	< 0.01[a]	0.51	0.25
FEA		0.73	0.03	0.38	0.48	0.65	0.09

[a] $MR^2 \geq 0.8$ ($r \geq 0.9$) and *p*-value < 0.05

[b] The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

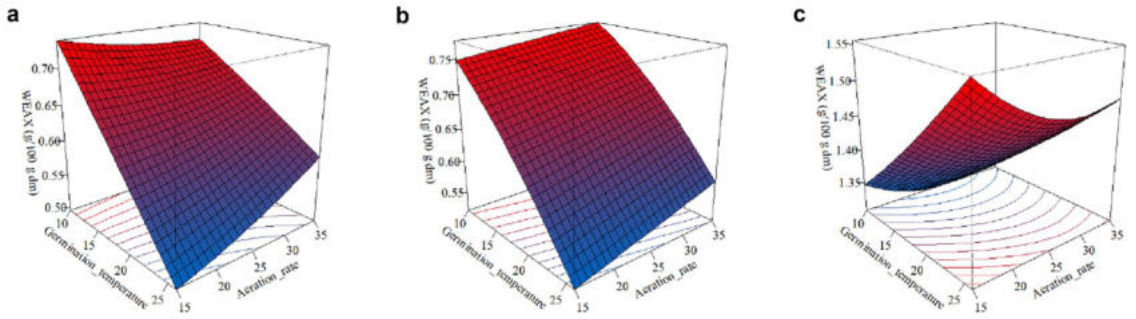


Fig. 3 Response surface plots for the impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the water-extractable arabinoxylan (WEAX) content in malts of the barley varieties Solist (**a**) and Quench (**b**) and the wheat variety Dacanto (**c**)

Similar to the steeping temperature, also in case of the germination temperature in combination with the aeration rate, the tendencies found for the water-extractable arabinoxylan contents could be linked to the dependency of the soluble and free phenolic acid contents on these parameters. For the barley varieties, also a decrease of the free phenolic acid contents with increasing germination temperatures was found, whereby the aeration rate played a secondary role (Figure S4 in the Online Resources). Exemplarily, comparing "favorable" (cinnamic acid reduction) and "unfavorable" (cinnamic acid increase) parameters, a reduction of free cinnamic, *p*-coumaric, and ferulic acid of roughly 70, 70, and 50% was found in malts of Solist within the present study (Fig. 4). Looking at the wheat variety Dacanto, free cinnamic and *p*-coumaric acid contents, again in accordance with the results of the water-extractable arabinoxylan contents, were unaffected by the varied parameters (Table 2). Ferulic acid was an exception to this rule and contrarily showed a decrease with increasing germination temperatures, which was similar to the barley varieties (Figure S4 in the Online Resources). In comparison to the free phenolic acid contents, the impact of the applied parameters on the total and soluble phenolic acid contents in the same malts was rather limited (Table 2).

Mashing of the malts produced at different germination temperatures in combination with the aeration rates led to wort with similar trends for the soluble and free phenolic acid contents as found for the respective malts (Figures S5 and S6 in the Online Resources). More precisely, this was the case for cinnamic and ferulic acid, whereas for *p*-coumaric acid no statistical correlation was found (Table 2). According to the results of the present study, sets of favorable (cinnamic acid decrease) parameters led to a reduction of soluble cinnamic acid in wort of approximately 52 and 40% in the varieties Solist and Dacanto compared to unfavorable (cinnamic acid increase) parameters. For soluble ferulic acid contents, a smaller reduction of 20 and 26% was found in the varieties Solist and Quench (Figure S5 in the Online Resources).

Thus, the results of the present study recommend higher germination temperatures for the preparation of barley and wheat malts for wheat beer brewing. Regarding the aeration

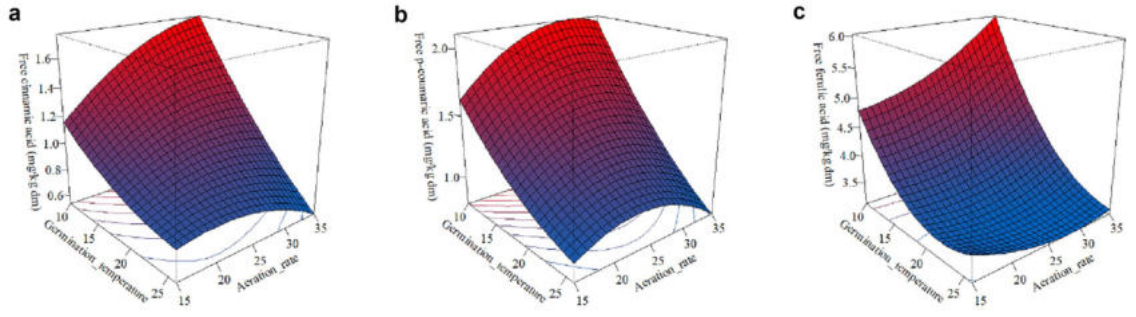


Fig. 4 Response surface plots for the impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the free contents of cinnamic acid (**a**), *p*-coumaric acid (**b**), and ferulic acid (**c**) in malts of the barley variety Solist

rate, no final recommendation can be made, as no clear trends were found among the phenolic acids in malt and wort and, apart from that, the aeration rate seems to play only a secondary role in the reduction of the undesired styrene precursor cinnamic acid in wheat beer. In conclusion, germination parameters revealed a high potential for styrene mitigation, since a reduction of the undesired cinnamic acid in wort of up to 50% was possible within the present study.

Impact of the withering temperature during kiln-drying on the release of phenolic acids during malting

Four different withering temperatures (30, 40, 50, and 60 °C; applied isothermally to enable the comparability of the data related to specific temperatures) were applied during malting of the barley varieties Solist and Quench and the wheat variety Dacanto to evaluate the impact of kiln-drying on the release of the precursors of the desired and undesired vinyl aromatics during malt production. Based on the temperature optima (varying between 40 and 70 °C) of the arabinoxylan-degrading enzymes [22] and of the feruloyl esterase [42], responsible for the direct release of phenolic acids from this non-starch polysaccharide structure, the withering temperature was expected to have the most pronounced impact on the release of the phenolic acids during malting among the malting parameters evaluated in the present study, as the temperatures applied during kiln-drying represent the range showing the biggest differences in the respective enzyme activity. In fact, the results of the present study confirmed this expectation (Fig. 5; for detailed values and statistics, see Tables S4–S6 in the Online Resources). In malt, free phenolic acid contents were found to be highest at a withering temperature of 60 °C and lowest at 30 °C. For the barley varieties Solist and Quench, a moderate increase was found for the free cinnamic, *p*-coumaric, and ferulic acid contents from 30 to 50 °C, followed by a dominant increase from 50 to 60 °C. Interestingly, the impact was more pronounced for cinnamic and *p*-coumaric acid as for ferulic acid. Comparing the amounts of free phenolic acids at 30 (favorable) and 60 (unfavorable) °C, the withering temperature had the power to decrease the contents of cinnamic acid by 76 and 84%, of *p*-coumaric acid by 72 and 80%, and of ferulic acid by 29 and 33% in malts of Solist and Quench (Fig. 5). For the wheat variety Dacanto, the increase of the free phenolic acid contents was found to be less distinct than for the barley varieties, especially the pronounced increase from 50 to 60 °C was not present in the wheat variety. Still, with respect to the analyzed wheat variety, decreases in the free phenolic acid contents of 67, 21, and 24% for cinnamic, *p*-coumaric, and ferulic acid were possible comparing the contents at 30 (favorable) and 60 (unfavorable) °C (Fig. 5).

Looking at the total and soluble phenolic acid contents in malts (Fig. 5), the impact of the withering temperature was less pronounced, which was in accordance with the results of the steeping temperature as well as of the germination temperature in combination with the aeration rate. At a first glance, this statement seemed to be in conflict with the pattern found for cinnamic acid; however, it was the consequence of the occurrence of cinnamic acid mostly in its free form. Also in a former study [35], the free form of cinnamic acid was the most dominant form in malts, which was explained by the fact that cinnamic acid only plays the role of an intermediate in the phenyl propanoid metabolism leading to the formation of further phenolic acids, e.g., *p*-coumaric, ferulic, and sinapic acid [14, 15].

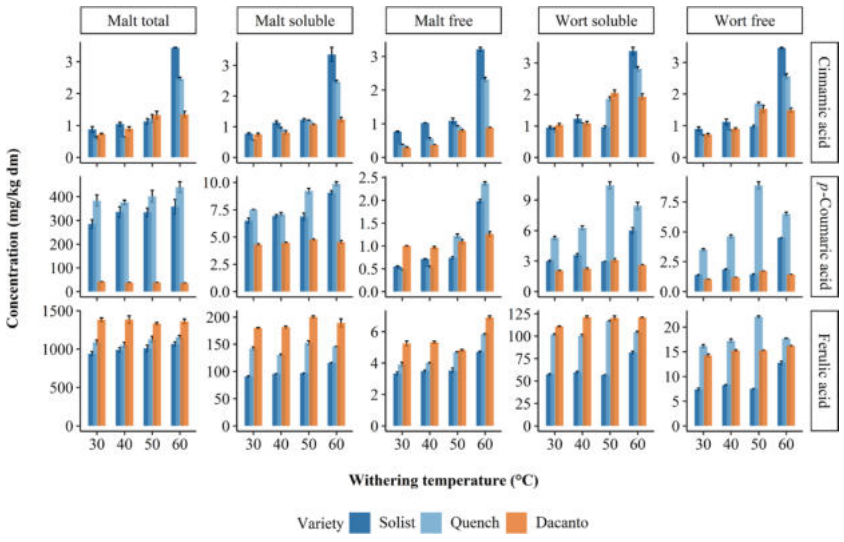


Fig. 5 Total (= sum of free, soluble ester-bound, and insoluble ester-bound), soluble (= sum of free and soluble ester-bound), and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid in dependency of the withering temperature (30, 40, 50, and 60 °C) in malts of the barley varieties Solist and Quench and the wheat variety Dacanto as well as in the corresponding wort (50 g of malt, 400 mL of water) produced thereof

A comparison of the total, soluble, and free cinnamic acid contents in malts (Fig. 5) further revealed that enzymatic degradation processes, leading to soluble and free phenolic acids, are not solely able to explain all the concentration changes found in dependency of the varied withering temperatures. As most of the differences found in the total and soluble contents were caused by the free form in both grain types, which was not the result of the feruloyl esterase activity as already explained above, proven by the absence of considerable amounts of insoluble or soluble ester-bound cinnamic acid contents, a formation of cinnamic acid via the phenyl propanoid metabolism is very likely. Therefore, also the biosynthesis of phenolic acids has to be considered to explain changes in phenolic acid contents caused by the different malting parameters.

To estimate to which extent arabinoxylan-degrading enzymes contribute to the release of phenolic acids based on the varied parameter, the water-extractable arabinoxylan content can be used. For the barley variety Solist, an increase of the water-extractable arabinoxylan content by about 25% from 0.39 to 0.49 g/100 g dm (Fig. 6) was found with an increasing temperature from 30 to 60 °C. For Quench, the temperature with the highest water-extractable arabinoxylan content was 50 °C (0.68 g/100 g dm), while the contents were at maximum 24% lower at the other temperatures. For the wheat variety Dacanto, statistical data revealed that the water-extractable arabinoxylan content was unaffected by the withering temperature (Table S6 in the Online Resources). Therefore, arabinoxylan-degrading enzymes cannot be used to explain the differences in the contents of > 24%. With overall differences of up to 84%, this fact underlines that arabinoxylan-degrading enzymes are only one of the factors explaining the overall changes. For the feruloyl esterase, the withering temperature had no effect on its activity in malts of the barley variety Solist (Fig. 6 and Table S4 in the Online Resources). In contrast, for both the barley variety Quench and the wheat variety Dacanto, the highest feruloyl esterase activity was found in malts withered at 50 °C with 7.63 and 3.87 U/g dm, respectively. Comparing the activities at 30, 40, and 60 °C to the temperature optimum of the enzyme at 50 °C, activities were < 41% lower.

With respect to the withering temperature, mashing of all three varieties led to wort with a similar pattern of soluble and free cinnamic acid contents compared to the applied malts (Fig. 5). For the barley varieties, the highest contents of cinnamic acid were found at 60 °C and for the wheat variety at 50 and 60 °C. Comparing the contents at a withering temperature of 30 (favorable) and 60 (unfavorable) °C, this parameter had the power to reduce the soluble cinnamic acid content by 72, 69, and 46% in the respective wort of Solist, Quench, and Dacanto. For Solist, also the desired precursors had their highest contents in wort produced from malts withered at 60 °C. Again, in accordance with the malts, only tiny differences were found between the withering temperatures of 30, 40, and 50 °C, whereat the most distinctive increase was found between 50 and 60 °C (Fig. 5). Therefore, in case of Solist, a correlation was given for the soluble and free contents between wort and the corresponding malts. Comparing the contents at 30, 40, and 50 °C to the content at 60 °C, a reduction of soluble cinnamic acid was accompanied by a reduction of *p*-coumaric and ferulic acid of up to 51 and 31%. With regard to the soluble and free *p*-coumaric and ferulic acid contents in wort of the barley variety Quench and the wheat variety Dacanto, the highest contents were found at a withering temperature of 50 °C (negligible for ferulic acid in Dacanto) (Fig. 5 and Table S6 in the Online Resources). This was quite different to the pattern found for the corresponding malts and seemed to be a consequence of the feruloyl esterase activity, still active after kiln-drying, which had its temperature optimum in the malts of Quench and Dacanto at 50 °C (Fig. 6). Another evidence for this assumption was the absence of this effect in the case of Solist, where no significant impact was found on the feruloyl esterase activity in dependency of the withering temperature (Table S4 in the Online Resources). Comparing unfavorable with favorable conditions related to cinnammic

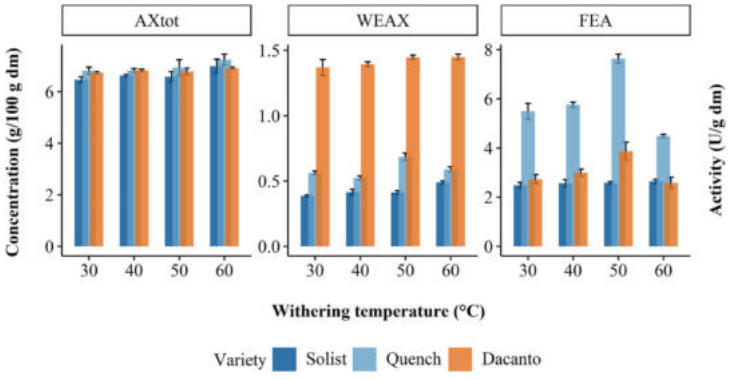


Fig. 6 Total (AXtot) and water-extractable arabinoxylan (WEAX) contents as well as feruloyl esterase activity (FEA) in dependency of the withering temperature (30, 40, 50, and 60 °C) in malts of the barley varieties Solist and Quench and the wheat variety Dacanto

acid reduction, also a reduction of the soluble desired *p*-coumaric and ferulic acid contents of up to 49 and 14% for Quench and 34 and 8% for Dacanto has to be considered.

From the perspective of styrene mitigation in wheat beer via its precursor cinnamic acid, low withering temperatures are recommended during malt production according to the results of the present study. Concerning the desired precursors, sufficient amounts allowing the formation of the distinct aroma in wheat beer brewed from these malts can still be expected, although favorable conditions could reduce the *p*-coumaric acid content by up to 51% and the ferulic acid content by up to 31%. With the power to reduce the soluble cinnamic acid content in wort by up to 72%, the withering temperature was proven to have the strongest impact on the formation and release of the desired and undesired precursors of the vinyl aromatics during malt production among the parameters analyzed within this study, underlining its importance as a tool for styrene mitigation.

Impact of the kilning temperature on the release of phenolic acids during malting

In a former study comparing dark and pale wheat beers [10], significantly lower concentrations of the desired and undesired vinyl aromatics were analyzed in dark wheat beers. Further, a nearly complete decarboxylation of the free precursors was found in both types. With these analyte pattern at hand and considering that both types were brewed with the same original gravity, it was assumed that phenolic acid contents in dark malts were significantly reduced via thermal decarboxylation as a consequence of the high temperature applied during kiln-drying. This assumption was based on the results of the study of Samaras et al. [43], who showed that high kilning temperatures, used for the production of dark malts, led to a massive decrease of the free phenolic acid contents in the respective malts. However, according to the results of the present study, thermal decarboxylation is only one of the factors responsible for the lower vinyl aromatic contents in dark wheat beers. Malts of the barley varieties Solist and Quench and the wheat variety Dacanto kilned at 80 (pale) and 200 (dark) °C revealed that the total, soluble, and free phenolic acid contents were only slightly affected by the higher kilning temperature of 200 °C compared to 80 °C (Fig. 7; for detailed values and statistics, see Tables S4–S6 in the Online Resources).

Different to the malts, soluble and free phenolic acid contents were highly affected in wort. However, the slightly lower contents in the malts were not enough to explain the significant reductions in the corresponding wort (Tables S4–S6 in the Online Resources). In contrast, the absence of additional biosynthesis that is normally found for cinnamic acid, unusual low transfer rates of already soluble phenolic acids, and the lack of an additional release of *p*-coumaric and ferulic acid by the feruloyl esterase during mashing were found to cause the low phenolic acid contents in wort. A hint for the explanation of this observation might be given by the missing feruloyl esterase activity in malts produced at 200 °C, suggesting that also other enzymes involved in the biosynthesis and the degradation of non-starch polysaccharides were affected by the high kilning temperatures applied. This finding fits also to the bad filtration behavior found during mashing of dark malts in the present study.

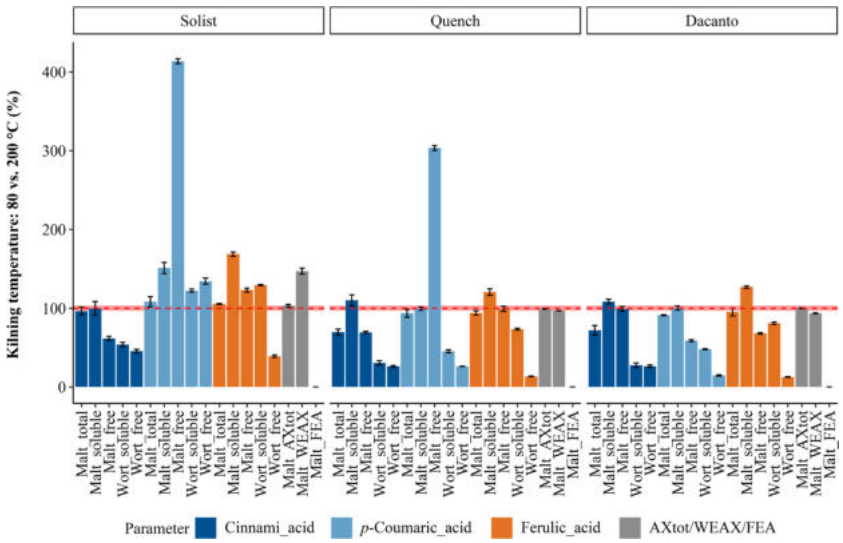


Fig. 7 Recovered total (= sum of free, soluble ester-bound, and insoluble ester-bound), soluble (= sum of free and soluble ester-bound), and free phenolic acid contents as well as total (AXtot) and water-extractable arabinoxylan (WEAX) contents and feruloyl esterase activity in dark malts (withering temperature, 50 °C; kilning temperature, 200 °C) in comparison to pale malts (withering temperature, 50 °C, kilning temperature, 80 °C) of the barley varieties Solist and Quench and the wheat variety Dacanto. The red line represents a recovery of 100%

Table 3 Malt analysis to determine the usability of the prepared malts for wheat beer brewing (green: parameter fulfills specifications, yellow: parameter within extended range, red: parameter outside of specifications)

Variety	Specification parameter[a]		Standard range[b]	Extended range[b]	Reference[c]	Steeping temperature (°C)			Germination temperature (°C)/aeration rate (L/min)									Withering temperature (°C)			
						10	20	30	10/25	12/18	12/32	18/15	18/25	18/35	24/18	24/32	26/25	30	40	50	60
	n[d] =				1	2	2	2	1	1	1	1	6	1	1	1	1	1	1	1	1
Solist	Moisture content	(%)	3.0 - 5.0	(up to 5.8)	5.6	4.3	4.5	4.5	4.7	4.5	4.6	4.7	4.6	4.8	4.7	4.9	4.5	5.7	5.1	4.8	4.7
	Extract[e]	(%)	79 - 82	(-)	80.8	79.7	79.8	79.5	83.7	83.3	82.9	81.5	81.6	81.4	80.8	81.5	80.5	79.1	79.4	80.0	80.4
	Color	(EBC)	≤ 4.0[f]	(4.1 - 5.0)	4.4	5.5	5.4	4.9	4.7	5.2	4.9	6.3	5.3	5.4	6.4	5.9	6.6	4.1	4.4	5.4	5.9
	pH-value	(-)	5.85 - 5.90	(5.60 - 6.20)	5.84	6.05	5.96	6.05	6.08	6.10	6.05	6.15	6.07	6.14	6.06	6.12	6.12	6.01	6.03	6.08	6.04
	Total protein[e]	(%)	10.5 - 11.5	(8.0 - 13.0)	9.9	11.0	11.2	11.3	10.9	10.7	10.7	11.4	10.8	10.7	11.4	11.2	11.7	11.9	11.6	11.5	11.4
	Kolbach index	(%)	35 - 45	(-)	41	40	40	39	50	49	48	41	42	43	43	41	39	34	36	39	42
	FAN[e]	(mg/100g)	120 - 160	(-)	129	126	124	105	209	202	188	138	145	152	150	125	120	115	122	143	147
	Sol. β-glucan[e]	(mg/100g)	50 - 1100	(-)	84	47	45	101	75	48	53	49	57	58	44	38	71	202	207	211	219
Quench	Moisture content	(%)	3.0 - 5.0	(up to 5.8)	5.6	4.3	4.4	4.6	4.5	4.3	4.6	4.5	4.5	4.8	4.6	4.8	4.5	5.1	4.7	4.4	4.2
	Extract[e]	(%)	79 - 82	(-)	80.8	81.7	80.7	81.8	84.5	83.9	83.9	81.6	82.2	81.8	81.3	79.1	80.3	80.4	80.7	81.6	81.9
	Color	(EBC)	≤ 4.0[f]	(4.1 - 5.0)	4.4	4.2	4.2	4.3	3.6	4.7	3.7	5.6	4.3	3.8	4.8	5.1	5.2	3.6	3.9	4.5	5.0
	pH-value	(-)	5.85 - 5.90	(5.60 - 6.20)	5.84	6.12	6.04	6.14	6.02	6.09	6.04	6.18	6.12	6.20	6.22	6.13	6.24	6.14	6.12	6.11	6.15
	Total protein[e]	(%)	10.5 - 11.5	(8.0 - 13.0)	9.9	9.4	9.6	9.4	9.0	9.1	9.1	9.4	9.1	9.1	9.4	9.2	9.5	10.4	10.1	10.2	10.2
	Kolbach index	(%)	35 - 45	(-)	41	38	37	36	54	48	48	37	38	39	36	37	35	36	36	35	36
	FAN[e]	(mg/100g)	120 - 160	(-)	129	91	90	79	199	173	158	101	106	109	89	89	77	98	104	108	113
	Sol. β-glucan[e]	(mg/100g)	50 - 1100	(-)	84	61	69	149	71	37	52	40	47	53	59	31	161	209	221	253	258
Dacanto	Moisture content	(%)	n.a.[g]	n.a.[g]	5.9	5.0	5.2	5.3	5.4	5.2	5.3	5.3	5.3	5.4	5.4	5.5	5.3	5.8	5.2	4.9	4.9
	Extract[e]	(%)	n.a.[g]	n.a.[g]	82.9	83.1	84.5	83.1	85.3	86.8	85.8	87.0	86.5	85.9	85.1	85.8	86.0	83.7	84.6	84.5	85.1
	Color	(EBC)	n.a.[g]	n.a.[g]	4	5.3	5.5	5.5	3.7	5.1	3.8	6.5	4.7	4.0	5.4	5.9	5.4	4.2	4.8	5.4	6.2
	pH-value	(-)	n.a.[g]	n.a.[g]	6	6.19	6.01	6.18	6.31	6.23	6.20	6.20	6.21	6.22	6.21	6.22	6.10	6.19	6.18	6.14	6.18
	Total protein[e]	(%)	n.a.[g]	n.a.[g]	12.3	12.4	12.5	12.4	12.2	12	12.2	12.5	12.1	11.7	12.6	12.4	12.8	12.9	12.5	12.5	12.4
	Kolbach index	(%)	n.a.[g]	n.a.[g]	37	38	39	54	32	48	35	58	48	45	53	55	54	41	44	43	45
	FAN[e]	(mg/100g)	n.a.[g]	n.a.[g]	70	57	63	65	67	85	66	78	69	69	64	67	62	64	73	68	75
	Sol. β-glucan[e]	(mg/100g)	n.a.[g]	n.a.[g]	43	67	61	68	62	55	67	61	66	67	48	53	72	65	63	67	64

[a]Average relative standard deviation in dependency of malting: moisture content 2.8%, extract 1.3%, color 5.6%, pH value 0.8%, total protein content 2.0%, Kolbach index 4.3%, FAN 6.6%, and soluble β-glucan content 8.6%

[b]Standard range and extended range of specifications for Pilsner type malts according to MEBAK® [30] and Krüger and Anger [44]

[c]Commercial Pilsner malt and wheat malt

[d]Malt replicates

[e]Values related to dry mass

[f]Referred to pale malt

[g]Specifications are not available for wheat malt

Specifications of applied malts

To determine to what extent these possible new styrene mitigation strategies can be applied to wheat beer brewing, malts were tested if they fulfill certain specifications. Addressing this challenge, malt samples prepared within this study were analyzed for the quality determining parameters moisture content, extract, color, pH value, total protein content, Kolbach index, FAN (free amino nitrogen), and soluble β-glucan content using the standard brewing methods described by MEBAK® [30] (Table 3). Malts prepared from the barley variety Solist matched the standard values defined by MEBAK® [30] and Krüger and Anger [44] in each case of the 16 different experimental settings. Only at a steeping temperature of 30 °C or a withering temperature of 30 °C, the FAN content was slightly too low (105 and 115 mg/100 g, respectively). For the barley variety Quench, again most of the parameters indicated that the respective malts had a high brewing potential. Indeed, the FAN values were too low to guarantee the sufficient sustenance of yeast with essential nutrients during fermentation. This seemed to be a characteristic of this variety and can also be explained by the lower total protein content. However, this should not limit the applicability of these malts at first instance, and thus, the significance of the gained knowledge for styrene mitigation, as it is a common practice in the malting industry to blend malts with different specifications to match the requirements of a distinct beer type.

Based on the fact that the standard malt quality values are defined for Pilsner malts, malts prepared from the wheat variety Dacanto were compared to a commercial wheat malt used for beer brewing, again showing high accordance of the values. Thus, also for the wheat malts prepared within this study, their potential for wheat beer brewing could be shown. In conclusion, malts prepared within this study showed a high conformance with standard malt quality values and values from commercial malts, highlighting their applicability for wheat beer brewing, and therefore, underlining malting as an important tool within a strategy for styrene mitigation.

Studies how malting parameters affect the content of phenolic acids in malts of barley and wheat are scarcely available. A study by Szwajgier et al. [45], addressing the impact of the pH value of the steeping water and the temperature applied during steeping and germination on the release of the free desired phenolic acids during malting, showed that a reduced pH value of the steeping water of pH 5.2 compared to 7.4 during malting of barley (varieties: Rudzik and Krona) resulted in an increase of the content of free *p*-coumaric and ferulic acid by approximately 200–300%. Further, they attested an increase of these phenolic acids by 160–210% when increasing the temperature during steeping and germination from 14 to 22 °C. However, these results were in conflict with a study of Langos et al. [24], who varied the germination temperature between 12 and 18 °C, with nearly no effect on the free phenolic acid contents in barley malts (variety: Marthe) (cinnamic, *p*-coumaric, and ferulic acid: 1.17, 1.17, and 3.24 mg/kg dm at 12 °C; 1.04, 1.15, and 3.29 mg/kg dm at 18 °C), and, in line with the results of the present study, with slightly decreasing contents with an increase of the germination temperature for wheat malts (variety: Hermann) (cinnamic, *p*-coumaric, and ferulic acid: 0.61, 0.84, and 2.54 mg/kg dm at 12 °C; 0.54, 0.69, and 2.41 mg/kg dm at 18 °C). Additionally, Langos et al. [24] showed that an prolongation of the germination time from 5 to 7 days led to an increase of the free phenolic acid contents in barley malts (cinnamic, *p*-coumaric, and ferulic acid: 0.85, 0.84, and 2.94 mg/kg dm at 5 days; 1.19, 1.07, and 3.50 mg/kg dm at 7 days), whereas no clear trend was found in wheat malts (cinnamic, *p*-coumaric, and ferulic acid: 0.45, 0.83, and 2.35 mg/kg dm at 5 days; 0.48, 0.65, and 2.68 mg/kg dm at 7 days).

In summary, the results of the present study proved the ability of the malting parameters steeping temperature, germination temperature in combination with the aeration rate, withering temperature, and kilning temperature to lower the input of the styrene precursor cinnamic acid into the brewing process. The lab-scale experiments also revealed promising perspectives regarding the malts prepared in this study fulfilled most of the malt quality specifications for brewing. With the capacity to lower the soluble cinnamic acid content in wort by up to 72%, the withering temperature during kiln-drying had the strongest impact among the malting parameters evaluated in the present study, followed by the germination temperature together with the aeration rate, showing a reduction capacity of up to 52%, and the steeping temperature having the lowest impact with a maximum reduction capacity of 16%. In dark wheat beers, the kilning temperature proved to be another important factor, with the capacity to reduce styrene also by up to 72%. Conditions, favorable for lower cinnamic acid contents, also had a reducing effect on the contents of the desired precursors. While *p*-coumaric acid derives mainly from barley malts, the potential regarding ferulic and cinnamic acid is quite equal to malts from barley and wheat [35]. However, the corresponding wort was found to have still sufficient amounts of *p*-coumaric and ferulic acid to allow the formation of the distinct aroma of wheat beer expected by the consumers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-021-03852-5.

Acknowledgements The authors thank Miss Anneliese Köhler for technical assistance, and Mr. Sami Kaviani-Nejad for performing the LC/MS–MS measurements.

Funding Open Access funding enabled and organized by Projekt DEAL. The IGF Project (AiF 19593 N) of the Association for the Promotion of Science of the German Brewing Industry (Wifö) was supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a decision by the German Bundestag.

Declarations

Conflict of interest None.

Compliance with ethics requirements The authors Valerian Kalb, Michael Granvogl, Torsten Seewald, and Thomas Hofmann hereby confirm that this manuscript is performed according and follows the COPE guidelines and has not already been published nor is it under consideration for publication elsewhere. This article does not contain any studies with human or animal subjects.

References

1. Shimada K, Kimura D, Yasui Y, Tanaka H, Matsushita S, Hagihara H, Nagakura M, Kawahisa M (1992) Styrene formation by the decomposition by *Pichia carsonii* of *trans*-cinnamic acid added to a ground fish product. Appl Environ Microbiol 5:1577–1582
2. Steele DH, Thornburg MJ, Stanley JS, Miller RR, Brooke R, Cushman JR, Cruzan G (1994) Determination of styrene in selected foods. J Agric Food Chem 42:1661–1665
3. Fragnière C, Aebischer J-N, Dudler V, Sager F (2003) A short study on the formation of styrene in cinnamon. Mitt Lebensm Hyg 94:609–620
4. Renner R, Bartels H, Tressl R (1976) Enrichment and quantitative determination of beer aroma substances by gas extraction (in German). Monatsschr Brau 29:478–480
5. Tressl R, Friese L, Fendesack F, Koeppler H (1978) Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. J Agric Food Chem 6:1422–1426
6. WHO (1996) Guidelines for drinking-water quality. World Health Organization, Geneva
7. IARC (2002) IARC monographs of the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer, Lyon
8. Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Hardy CJ, Coombs DW, Mullins PA, Brown WR (1998) Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. Toxicol Sci 46:266–281
9. Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Bevan C, Hardy CJ, Coombs DW, Mullins PA, Brown WR (2001) Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. J Appl Toxicol 21:185–198
10. Kalb V, Granvogl M (2019) Formation of desired smoky key odorants in wheat beer: a comparison with the undesired toxicologically relevant styrene. In: Guthrie B, Beauchamp JD, Buettner A, Toth S, Qian MC (eds) Sex, smoke, and spirits: the role of chemistry; ACS Symposium Series 1321. American Chemical Society, Washington, DC
11. Langos D, Granvogl M (2016) Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. J Agric Food Chem 11:2325–2332
12. Daly B, Collins F, Madigan D, Donelly D, Coakley M, Ross P (1997) An investigation into styrene in beer. Proc Congr Eur Brew Conv 26:623–630
13. Langos D, Granvogl M, Schieberle P (2013) Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. J Agric Food Chem 47:11303–11311
14. Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40:347–369
15. Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. Biotechnol J 10:1214–1234
16. Finche GB (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annu Rev Plant Physiol Plant Mol Biol 40:305–346
17. Fincher GB (2009) Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. Plant Physiol 1:27–37
18. Gubler F, Ashford AE (1985) Release of ferulic acid esters from barley aleurone. I. Time course of gibberellic-acid-induced release from isolated layers. Aust J Plant Physiol 3:297–305
19. Dvořáková M, Guido LF, Dostálek P, Skulilová Z, Moreira MM, Barros AA (2008) Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. J Inst Brew 1:27–33
20. Maillard M-N, Berset C (1995) Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. J Agric Food Chem 7:1789–1793
21. Nordkvist E, Salomonsson A-C, Åman P (1984) Distribution of insoluble bound phenolic acids in barley grain. J Sci Food Agric 35:657–661
22. Rasmussen CV, Hansen HB, Hansen Å, Larsen LM (2001) pH-, temperature- and time-dependent activities of endogenous endo-β-D-xylanase, β-D-xylosidase and α-L-arabinofuranosidase in extracts from ungerminated rye (*Secale cereale* L.) grain. J Cereal Sci 1:49–60
23. Langos D, Gastl M, Granvogl M (2017) Reduction of toxicologically relevant styrene in wheat beer using specially produced wheat and barley malts. Eur Food Res Technol 193:558–568
24. Langos D, Granvogl M, Gastl M, Schieberle P (2015) Influence of malt modifications on the concentrations of free phenolic acids in wheat and barley malts. Brew Sci 68:93–101
25. Schwarz KJ, Methner F-J (2011) Styrene concentrations during wheat beer production. Brew Sci 64:156–158
26. Schwarz KJ, Boitz LI, Methner F-J (2012) Enzymatic formation of styrene during wheat beer fermentation is dependent on pitching rate and cinnamic acid content. J Inst Brew 3:280–284
27. Schwarz KJ, Boltz LI, Methner F-J (2012) Release of phenolic acids and amino acids during mashing dependent on temperature, pH, time and raw materials. J Am Soc Brew Chem 70:290–295
28. Schwarz KJ, Stübner R, Methner F-J (2012) Formation of styrene dependent on fermentation management during wheat beer production. Food Chem 4:2121–2125

29. Schwarz KJ, Boltz LI, Methner F-J (2013) Influence of mashing conditions on the release of precursors of phenolic wheat beer aroma (in German). Brauwelt 153:274–279
30. Pfenninger H (1997) Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK). Selbstverlag der MEBAK, Freising
31. European Brewery Convention (2010) Analytica-EBC. Fachverlag Hans Carl, Nuremberg
32. Kalb V, Seewald T, Hofmann T, Granvogl M (2020) The role of endogeneous enzymes during malting of barley and wheat varieties in the mitigation of styrene in wheat beer. J Agric Food Chem 68:13888–13896
33. Andersson AAM, Lampi A-M, Nyström L, Piironen V, Li L, Ward JL, Gebruers K, Courtin CM, Delcour JA, Boros D, Fraś A, Dynkowska W, Rakszegi M, Bedo Z, Shewry PR, Åman P (2008) Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN diversity screen. J Agric Food Chem 21:9767–9776
34. Gebruers K, Dornez E, Boros D, Fraś A, Dynkowska W, Bedo Z, Rakszegi M, Delcour JA, Courtin CM (2008) Variation in the content of dietary fiber and components thereof in wheats in the HEALTHGRAIN diversity screen. J Agric Food Chem 21:9740–9749
35. Kalb V, Seewald T, Hofmann T, Granvogl M (2020) Studies on the impact of malting and mashing on the free, soluble ester-bound, and insolbule ester-bound forms of desired and undesired phenolic acids aiming at styrene mitigation during wheat beer brewing. J Agric Food Chem 68:12421–12432
36. R Development Core Team (2019) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.r-project.org. Accessed 14 Apr 2020
37. RStudio Team (2019) RStudio: integrated development for R. RStudio, Boston, MA. https://www.rstudio.com/. Accessed 14 Apr 2020
38. Ogle D, Wheeler P, Dinno A (2020) R FSA: simple fisheries stock assessment methods. https://cran.r-project.org/package=FSA. Accessed 14 Apr 2020
39. Fox J, Weisberg S (2019) R car: companion to applied regression. https://cran.r-project.org/package=car. Accessed 14 Apr 2020
40. Lenth R (2018) R rsm: response-surface analysis. https://cran.r-project.org/package=rsm. Accessed 14 Apr 2020
41. Soetaert K (2019) R plot3D: plotting multi-dimensional data. https://cran.r-project.org/package=plot3D. Accessed 14 Apr 2020
42. Wang D, Meng-Meng G, Jin-Hua D (2011) Determination and characterisation of feruloyl esterase in wheats and malts. Food Ferment Ind 37:181–185
43. Samaras TS, Camburn PA, Chandra SX, Gordon MH, Ames JM (2005) Antioxidant properties of kilned and roasted malts. J Agric Food Chem 20:8068–8074
44. Krüger E, Anger H-M (1990) Kennzahlen zur Betriebskontrolle und Qualitätsbeschreibung in der Brauwirtschaft: Daten über Roh- und Hilfsstoffe, Halbfertig- und Fertigprodukte bei der Bierbereitung. Behr, Hamburg
45. Szwajgier D, Pielecki J, Targoński Z (2005) Changes of free ferulic and coumaric acid contents during malting of balrey grain. Pol J Food Nutr Sci 4:423–429

Supplementary Material

for

The malting parameters steeping, germination, withering, and kilning temperature and aeration rate as possibilities for styrene mitigation in wheat beer

Valerian Kalb[1], *Torsten Seewald*[2], *Thomas Hofmann*[1,3], *and Michael Granvogl*[4,*]

[1] Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, 85354 Freising, Germany

[2] Institut für Lebensmitteltechnologie und Lebensmittelchemie, Fachgebiet Brauwesen, Technische Universität Berlin, 13353 Berlin, Germany

[3] Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München, Lise-Meitner-Straße 34, 85354 Freising, Germany

[4] Fachgebiet Lebensmittelchemie und Analytische Chemie (170a), Institut für Lebensmittelchemie, Fakultät Naturwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany

---

*Corresponding Author
Phone: +49 711 459 23979
Fax: +49 711 459 24096
E-mail: michael.granvogl@uni-hohenheim.de

FIGURE CAPTIONS

Fig. S1 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the water-extractable arabinoxylan content and the feruloyl esterase activity in the barley malts Solist and Quench and in the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and p-value < 0.05)

Fig. S2 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the total contents of cinnamic, *p*-coumaric, and ferulic acid in the barley malts Solist and Quench and in the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and p-value < 0.05)

Fig. S3 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the soluble contents of cinnamic, *p*-coumaric, and ferulic acid in the barley malts Solist and Quench and in the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and p-value < 0.05)

Fig. S4 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the free contents of cinnamic, *p*-coumaric, and ferulic acid in the barley malts Solist and Quench and in the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and p-value < 0.05)

Fig. S5 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the soluble contents of cinnamic, *p*-coumaric, and ferulic acid in wort (50 g of malt, 400 mL of water) produced from the barley malts Solist and Quench and the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and p-value < 0.05)

Fig. S6 Response surface modeling: impact of the germination temperature (°C) in combination with the aeration rate (L/min) on the free contents of cinnamic, *p*-coumaric, and ferulic acid in wort (50 g of malt, 400 mL of water) produced from the barley malts Solist and Quench and the wheat malt Dacanto ($^{a}MR^2 \geq 0.8$ ($r \geq 0.9$) and *p*-value < 0.05)

Table S1 Impact of the steeping temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Solist and the corresponding wort

Process step	Type	T (°C)	Concentrations (mg/kg dry mass)[a]											
			Cinnamic acid				*p*-Coumaric acid				Ferulic acid			
Malt	Total	10	1.92	(±7)	a		306	(±9)	a		941	(±4)	a	
		20	1.95	(±7)	a		310	(±8)	a		976	(±3)	a	
		30	1.97	(±2)	a		273	(±8)	a		968	(±5)	a	
	Soluble	10	1.72	(±6)	a	b	9.05	(±2)	a		130	(±5)	a	
		20	1.63	(±7)	a		8.92	(±3)	a		137	(±3)		
		30	1.80	(±6)		b	8.29	(±2)			125	(±2)	a	
	Free	10	0.972	(±6)	a		1.42	(±4)	a		4.27	(±3)	a	
		20	0.930	(±8)	a		1.42	(±3)	a		4.35	(±2)	a	
		30	0.954	(±5)	a		1.30	(±3)			4.03	(±2)		
Wort[b]	Soluble	10	2.48	(±6)	a		10.9	(±4)	a		104	(±6)	a	
		20	2.29	(±8)	a		11.8	(±1)			107	(±3)	a	
		30	2.16	(±25)	a		10.5	(±9)	a		95.0	(±2)		
	Free	10	2.05	(±5)	a		9.50	(±7)	a		27.1	(±12)	a	b
		20	2.03	(±8)	a		10.7	(±1)			31.7	(±2)	a	
		30	1.76	(±28)	a		9.01	(±11)	a		21.3	(±4)		b
			(U/g dry mass) [a]				Concentrations (g/100 g dry mass) [a]							
			FEA				AXtot				WEAX			
Malt		10	7.27	(±12)	a	b	7.01	(±3)	a		0.61	(±8)	a	
		20	8.04	(±5)	a		6.93	(±3)	a		0.64	(±4)	a	
		30	7.24	(±3)		b	6.90	(±2)	a		0.56	(±3)		

[a]Mean values of triplicates with relative standard deviations (%) in parentheses (technical replicates n = 2)

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

Values with no common letters differ significantly at the 95% confidence level

Table S2 Impact of the steeping temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Quench and corresponding wort

Process step	Type	T (°C)	Concentrations (mg/kg dry mass)[a]											
			Cinnamic acid			*p*-Coumaric acid			Ferulic acid					
Malt	Total	10	1.28	(±5)		372	(±6)	a	942	(±5)	a			
		20	1.17	(±3)		354	(±6)	a	b	917	(±5)	a		
		30	1.09	(±1)		332	(±3)		b	918	(±4)	a		
	Soluble	10	1.25	(±5)		8.53	(±2)	a		133	(±2)	a		
		20	1.05	(±6)	a	8.43	(±5)	a		133	(±3)	a		
		30	1.11	(±8)	a	7.97	(±3)			126	(±2)			
	Free	10	0.717	(±6)		1.08	(±4)			4.12	(±3)			
		20	0.627	(±8)		1.00	(±7)			3.99	(±3)			
		30	0.495	(±5)		0.87	(±3)			3.52	(±2)			
Wort[b]	Soluble	10	1.96	(±7)	a	9.39	(±2)	a		105	(±2)	a		
		20	1.76	(±9)	a	b	9.99	(±4)			106	(±2)	a	
		30	1.64	(±12)		b	9.18	(±1)	a		94.0	(±2)		
	Free	10	1.74	(±6)			7.85	(±2)	a		23.3	(±4)	a	b
		20	1.35	(±12)	a		8.75	(±2)			25.0	(±3)	a	
		30	1.28	(±6)	a		7.91	(±4)	a		20.0	(±1)		b
			(U/g dry mass) [a]				Concentrations (g/100 g dry mass) [a]							
			FEA				AXtot				WEAX			
Malt		10	6.90	(±2)	a		6.73	(±2)	a		0.68	(±1)		
		20	5.99	(±12)	a		6.84	(±2)	a		0.64	(±2)		
		30	6.79	(±5)	a		6.77	(±1)	a		0.58	(±1)		

[a]Mean values of triplicates with relative standard deviations (%) in parentheses (technical replicates n = 2)

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

Values with no common letters differ significantly at the 95% confidence level

Table S3 Impact of the steeping temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Dacanto and corresponding wort

Process step	Type	T (°C)	Concentrations (mg/kg dry mass)[a]											
			Cinnamic acid				*p*-Coumaric acid				Ferulic acid			
Malt	Total	10	1.28	(±7)	a		32.5	(±6)	a		1100	(±4)	a	
		20	1.44	(±2)			39.1	(±8)			1100	(±2)	a	
		30	1.32	(±7)	a		33.3	(±5)	a		1140	(±3)	a	
	Soluble	10	1.31	(±11)	a		4.64	(±11)	a		168	(±3)	a	
		20	1.42	(±10)	a		6.18	(±16)			190	(±11)	a	
		30	1.37	(±8)	a		4.85	(±3)	a		170	(±2)	a	
	Free	10	0.538	(±7)	a		0.951	(±12)	a		3.86	(±7)	a	
		20	0.673	(±12)		b	1.33	(±21)		b	4.46	(±2)		
		30	0.605	(±4)	a	b	1.05	(±6)	a	b	4.05	(±2)	a	
Wort[b]	Soluble	10	1.80	(±6)	a	b	2.70	(±17)			108	(±6)	a	
		20	1.69	(±13)	a		4.83	(±25)	a		126	(±12)	a	b
		30	1.96	(±7)		b	3.70	(±1)	a		121	(±2)		b
	Free	10	1.18	(±11)	a	b	1.56	(±25)			16.4	(±18)	a	
		20	1.06	(±17)	a		2.98	(±24)	a		16.9	(±11)	a	
		30	1.33	(±8)		b	2.46	(±6)	a		19.3	(±3)	a	
			(U/g dry mass)[a]				Concentrations (g/100 g dry mass)[a]							
			FEA				AXtot				WEAX			
Malt		10	3.96	(±4)	a		6.48	(±1)	a		1.36	(±3)	a	
		20	3.26	(±9)			6.64	(±1)		b	1.56	(±9)		b
		30	4.21	(±7)	a		6.57	(±2)	a	b	1.43	(±2)	a	b

[a]Mean values of triplicates with relative standard deviations (%) in parentheses (technical replicates n = 2)

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

Values with no common letters differ significantly at the 95% confidence level

Table S4 Impact of the withering temperature and kilning temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Solist and corresponding wort

Process step	Type	T [°C]	Concentrations (mg/kg dry mass)[a] Cinnamic acid			*p*-Coumaric acid			Ferulic acid		
Malt	Total	30/80	0.880	(±9)		284	(±6)	a	941	(±3)	a
		40/80	1.04	(±5)	a	335	(±7)	a b	990	(±3)	a b
		50/80	1.13	(±8)	a	334	(±5)	a b	1010	(±4)	a b
		60/80	3.43	(±0)		358	(±8)	b	1070	(±3)	b
		50/200	1.09	(±5)	a	361	(±6)	b	1060	(±1)	b
	Soluble	30	0.776	(±3)		6.50	(±4)	a	90.7	(±1)	
		40	1.13	(±5)	a	6.93	(±2)	a	95.5	(±0)	a
		50	1.22	(±4)	a	6.88	(±5)	a	96.4	(±1)	a
		60	3.35	(±7)		9.07	(±2)		116	(±0)	
		50/200	1.22	(±9)	a	10.4	(±5)		163	(±2)	
	Free	30/80	0.761	(±2)	a	0.545	(±3)		3.34	(±3)	a
		40/80	1.02	(±0)	b	0.713	(±1)	a	3.48	(±2)	a
		50/80	1.09	(±7)	b	0.741	(±3)	a	3.53	(±4)	a
		60/80	3.21	(±2)		1.98	(±2)		4.71	(±1)	
		50/200	0.671	(±4)	a	3.06	(±1)		4.34	(±2)	
Wort[b]	Soluble	30/80	0.945	(±5)	a	3.01	(±3)	a	57.4	(±1)	a
		40/80	1.23	(±10)		3.59	(±4)	b	59.9	(±2)	
		50/80	0.964	(±4)	a	2.95	(±1)	a	56.8	(±0)	a
		60/80	3.38	(±3)		6.02	(±5)		81.8	(±2)	
		50/200	0.519	(±5)		3.61	(±2)	b	73.4	(±1)	
	Free	30/80	0.899	(±7)	a	1.38	(±3)	a	7.41	(±3)	a
		40/80	1.12	(±8)		1.85	(±3)	b	8.27	(±1)	
		50/80	0.976	(±4)	a	1.45	(±3)	a	7.49	(±1)	a
		60/80	3.45	(±1)		4.49	(±0)		12.8	(±2)	
		50/200	0.445	(±5)		1.95	(±3)	b	2.92	(±4)	
			(U/g dry mass)[a] FEA			Concentrations (g/100 g dry mass)[a] AXtot			WEAX		
Malt		30/80	2.48	(±5)	a	6.46	(±2)	a	0.39	(±2)	a
		40/80	2.57	(±6)	a	6.62	(±1)	a b	0.41	(±5)	a
		50/80	2.58	(±2)	a	6.58	(±3)	a b	0.41	(±4)	a
		60/80	2.64	(±3)	a	6.99	(±4)	b	0.49	(±3)	
		50/200	<LoD[c]			6.79	(±2)	a b	0.60	(±3)	

[a]Mean values of triplicates with relative standard deviations (%) in parentheses

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

[c]LoD = 0.51 U/g dry mass

Values with no common letters differ significantly at the 95% confidence level

Table S5 Impact of the withering temperature and kilning temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Quench and corresponding wort

Process step	Type	T [°C]	Concentrations (mg/kg dry mass)[a] Cinnamic acid			*p*-Coumaric acid			Ferulic acid		
Malt	Total	30/80	0.606	(±8)	a	383	(±6)	a b	1090	(±3)	a b
		40/80	0.630	(±2)	a	376	(±3)	a	1050	(±3)	a
		50/80	1.22	(±10)		401	(±6)	a b	1130	(±3)	a b
		60/80	2.46	(±2)		440	(±5)	b	1160	(±2)	b
		50/200	0.847	(±5)		376	(±6)	a	1060	(±3)	a
	Soluble	30/80	0.561	(±0)		7.51	(±0)		142	(±2)	a
		40/80	0.923	(±6)		7.11	(±2)		130	(±1)	
		50/80	1.20	(±2)		9.22	(±3)	a	152	(±2)	b
		60/80	2.46	(±2)		9.88	(±2)		145	(±0)	a b
		50/200	1.32	(±6)		9.17	(±2)	a	184	(±3)	
	Free	30/80	0.377	(±4)		0.475	(±1)		3.91	(±3)	a
		40/80	0.551	(±6)		0.536	(±4)		3.99	(±1)	a
		50/80	0.921	(±3)		1.22	(±3)		4.68	(±1)	b
		60/80	2.31	(±3)		2.37	(±2)		5.82	(±1)	
		50/200	0.638	(±2)		3.70	(±1)		4.65	(±3)	b
Wort[b]	Soluble	30/80	0.871	(±8)		5.28	(±3)		102	(±1)	a
		40/80	1.09	(±1)		6.26	(±3)		101	(±1)	a
		50/80	1.86	(±4)		10.4	(±3)		117	(±1)	
		60/80	2.82	(±2)		8.41	(±4)		104	(±1)	
		50/200	0.568	(±9)		4.73	(±4)		85.6	(±2)	
	Free	30/80	0.693	(±4)		3.51	(±3)		16.1	(±2)	
		40/80	0.85	(±1)		4.62	(±3)	a	17.2	(±2)	
		50/80	1.70	(±3)		8.88	(±3)		22.1	(±1)	
		60/80	2.56	(±3)		6.49	(±2)	a	17.7	(±1)	
		50/200	0.445	(±4)		2.33	(±1)		2.96	(±3)	
			(U/g dry mass)[a] FEA			Concentrations (g/100 g dry mass)[a] AXtot			WEAX		
Malt		30/80	5.49	(±6)	a	6.81	(±2)	a	0.56	(±3)	a
		40/80	5.75	(±2)	a	6.82	(±1)	a	0.52	(±3)	
		50/80	7.63	(±2)		6.93	(±4)	a	0.68	(±4)	b
		60/80	4.49	(±2)		7.24	(±3)	a	0.59	(±3)	a
		50/200	<LoD[c]			6.88	(±1)	a	0.66	(±0)	b

[a]Mean values of triplicates with relative standard deviations (%) in parentheses

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

[c]LoD = 0.51 U/g dry mass

Values with no common letters differ significantly at the 95% confidence level

Table S6 Impact of the withering temperature and kilning temperature on the total, soluble, and free phenolic acid contents of cinnamic, *p*-coumaric, and ferulic acid as well as on the total arabinoxylan (AXtot) and water-extractable arabinoxylan (WEAX) concentrations and the feruloyl esterase activity (FEA) in malts of the barley variety Dacanto and corresponding wort

Process step	Type	T [°C]	Concentrations (mg/kg dry mass)[a] Cinnamic acid			*p*-Coumaric acid			Ferulic acid		
Malt	Total	30/80	0.730	(±3)	a	41.3	(±2)		1380	(±2)	a
		40/80	0.899	(±7)	a b	38.1	(±1)	a	1380	(±4)	a
		50/80	1.33	(±9)	c	38.0	(±3)	a	1330	(±1)	a
		60/80	1.35	(±7)	c	36.1	(±2)	b	1360	(±2)	a
		50/200	0.954	(±8)	b	34.6	(±1)	b	1270	(±5)	a
	Soluble	30/80	0.750	(±5)	a	4.31	(±2)	a	180	(±1)	a
		40/80	0.809	(±8)	a	4.47	(±2)	a b	181	(±1)	a
		50/80	1.07	(±1)	b	4.77	(±1)		200	(±1)	b
		60/80	1.23	(±6)	c	4.54	(±3)	b	189	(±4)	a b
		50/200	1.16	(±3)	b c	4.78	(±3)		254	(±1)	b
	Free	30/80	0.294	(±7)		0.999	(±1)	a	5.25	(±3)	a
		40/80	0.375	(±2)		0.966	(±2)	a	5.32	(±1)	a
		50/80	0.803	(±4)	a	1.10	(±4)		4.81	(±1)	
		60/80	0.883	(±1)		1.26	(±4)		6.90	(±1)	
		50/200	0.795	(±3)	a	0.644	(±2)		3.27	(±1)	
Wort[b]	Soluble	30/80	1.03	(±4)	a	2.05	(±3)		111	(±1)	
		40/80	1.09	(±5)	a	2.24	(±4)		121	(±1)	a
		50/80	2.05	(±4)	b	3.10	(±5)		120	(±2)	a
		60/80	1.93	(±5)	b	2.62	(±1)		121	(±0)	a
		50/200	0.566	(±10)		1.49	(±1)		97.6	(±2)	
	Free	30/80	0.715	(±5)		1.03	(±2)		14.3	(±2)	
		40/80	0.902	(±4)		1.18	(±2)		15.2	(±1)	a
		50/80	1.53	(±8)	a	1.71	(±1)		15.3	(±0)	a
		60/80	1.49	(±5)	a	1.42	(±2)		16.2	(±1)	
		50/200	0.403	(±6)		0.250	(±5)		1.93	(±3)	
			(U/g dry mass)[a]			Concentration (g/100 g dry mass)[a]					
			FEA			AXtot			WEAX		
Malt		30/80	2.73	(±7)	a	6.74	(±1)	a	1.37	(±4)	a b
		40/80	3.00	(±5)	a	6.82	(±1)	a	1.39	(±1)	a b
		50/80	3.87	(±9)		6.78	(±2)	a	1.45	(±1)	a
		60/80	2.58	(±9)	a	6.91	(±1)	a	1.45	(±2)	a
		50/200	<LoD[c]			6.76	(±0)	a	1.35	(±0)	b

[a]Mean values of triplicates with relative standard deviations (%) in parentheses

[b]Phenolic acid concentrations in the corresponding wort produced from 50 g of malt and 400 mL of water. The total content was not determined due to the absence of insoluble ester-bound phenolic acids in the wort

[c]LoD = 0.51 U/g dry mass

Values with no common letters differ significantly at the 95% confidence level

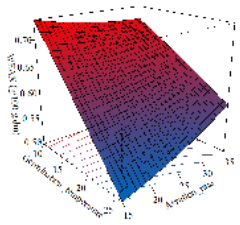
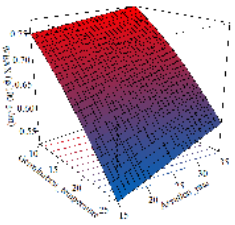
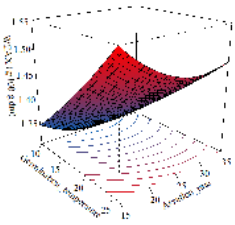
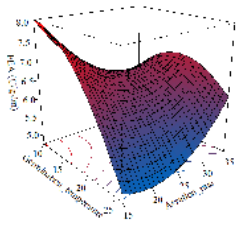
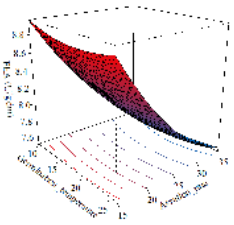
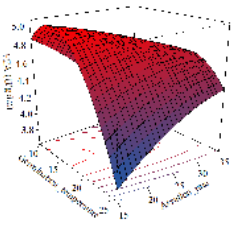
	Solist	Quench	Dacanto
Water-extractable arabinoxylan (g/100 g dry mass)			
	0.952*[a] | < 0.001*[a]	0.992*[a] | < 0.001*[a]	0.510 | 0.248
Feruloyl esterase activity (U/g dry mass)			
	0.732 | 0.032*[a]	0.383 | 0.478	0.646 | 0.086

Fig. S1

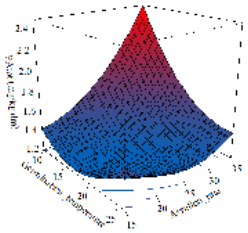
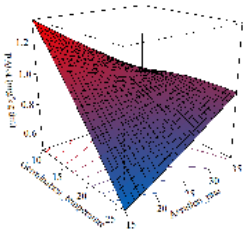
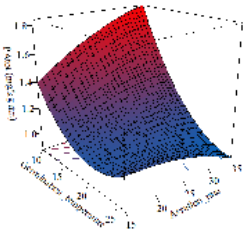
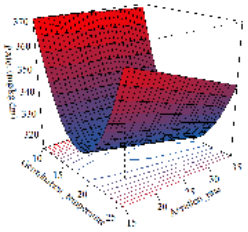
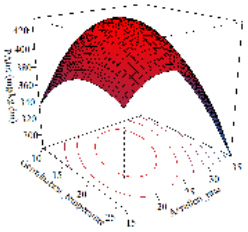
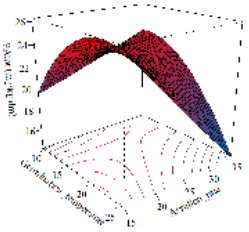
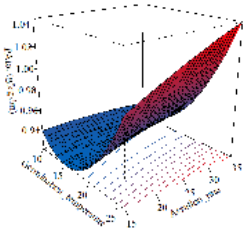
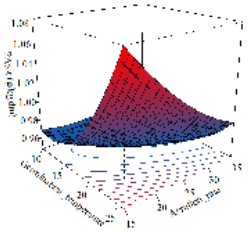
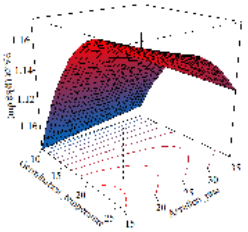
	Solist		Quench		Dacanto	
Cinnamic acid (mg/kg dry mass)		0.651 | 0.082		0.449 | 0.351		0.582 | 0.150
p-Coumaric acid (mg/kg dry mass)		0.410 | 0.425		0.385 | 0.473		0.537 | 0.208
Ferulic acid (g/kg dry mass)		0.681 | 0.060		0.679 | 0.061		0.573 | 0.161

Fig. S2

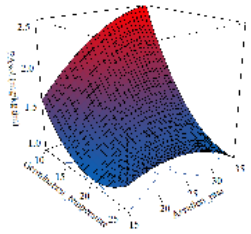
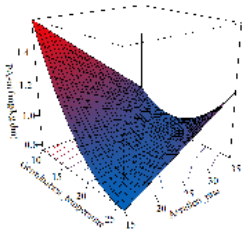
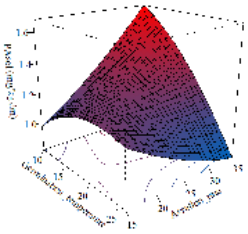
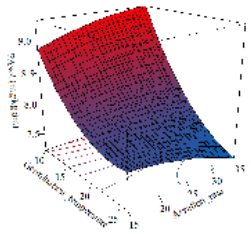
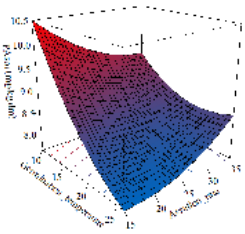
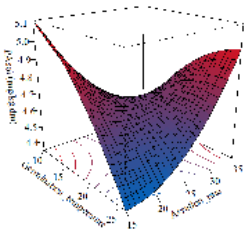
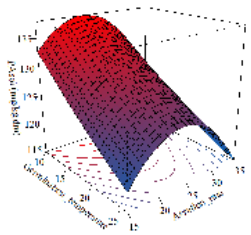
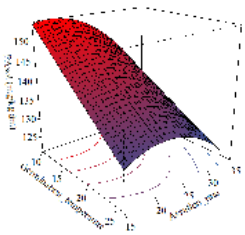
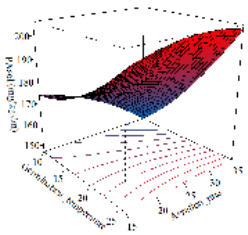
	Solist		Quench		Dacanto	
Cinnamic acid (mg/kg dry mass)						
	0.917* [a]	< 0.001* [a]	0.728	0.034* [a]	0.570	0.165
p-Coumaric acid (mg/kg dry mass)						
	0.741	0.029* [a]	0.616	0.114	0.206	0.827
Ferulic acid (mg/kg dry mass)						
	0.301	0.654	0.428	0.390	0.556	0.182

Fig. S3

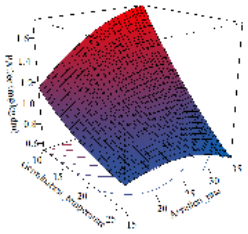
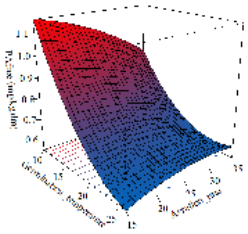
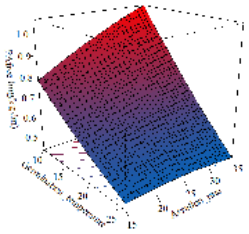
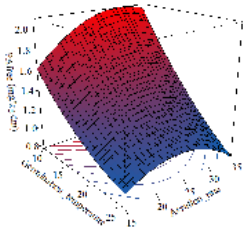
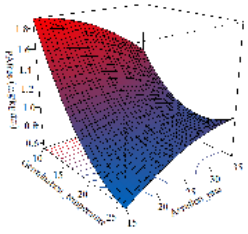
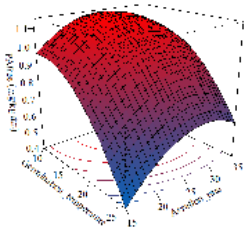
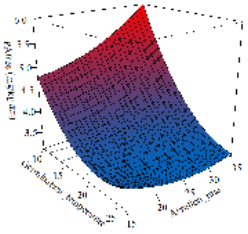
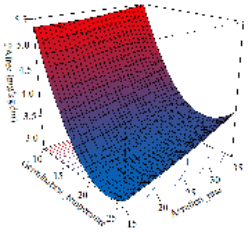
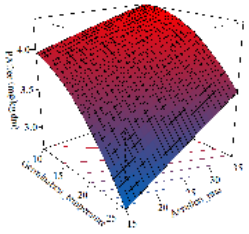
	Solist		Quench		Dacanto	
Cinnamic acid (mg/kg dry mass)						
	0.851*[a]	0.004*[a]	0.585	0.147	0.072	0.040*[a]
p-Coumaric acid (mg/kg dry mass)						
	0.800*[a]	0.011*[a]	0.763	0.021*[a]	0.675	0.064
Ferulic acid (mg/kg dry mass)						
	0.927*[a]	0.001*[a]	0.917*[a]	0.001*[a]	0.800*[a]	0.012*[a]

Fig. S4

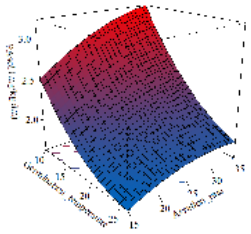
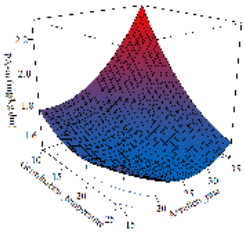
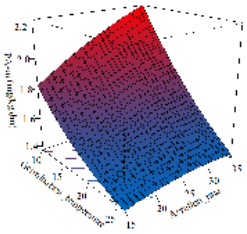
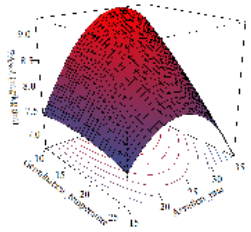
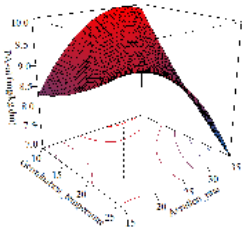
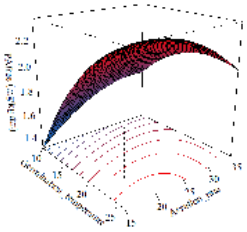
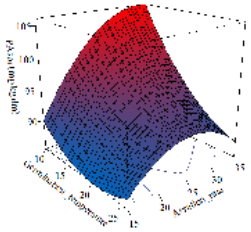
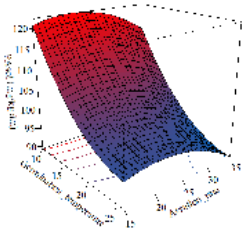
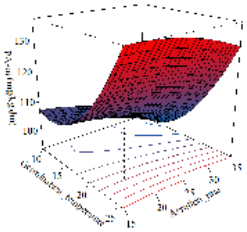
	Solist		Quench		Dacanto	
Cinnamic acid (mg/kg dry mass)						
	0.846* [a]	0.004* [a]	0.417	0.412	0.863* [a]	0.003* [a]
p-Coumaric acid (mg/kg dry mass)						
	0.475	0.306	0.354	0.538	0.311	0.625
Ferulic acid (mg/kg dry mass)						
	0.794	0.012* [a]	0.813* [a]	0.009* [a]	0.718	0.039* [a]

Fig. S5

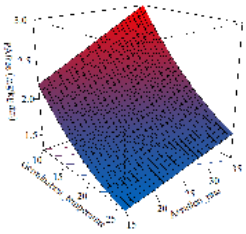
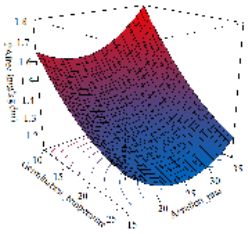
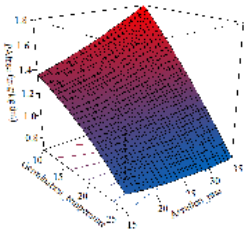
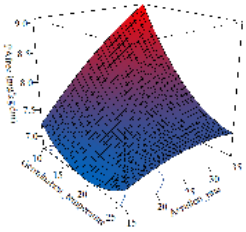
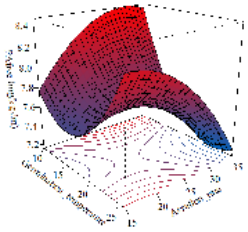
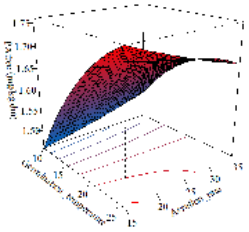
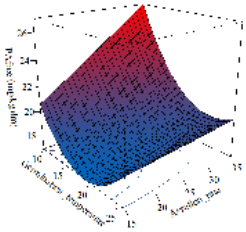
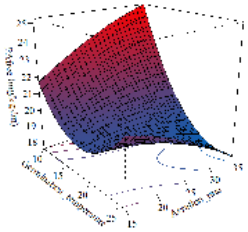
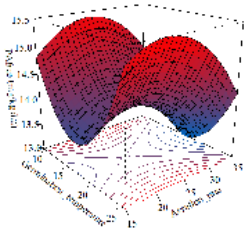
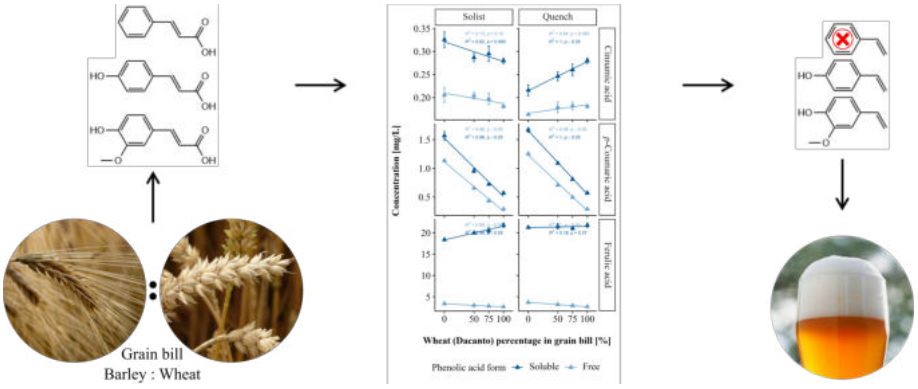
	Solist		Quench		Dacanto	
Cinnamic acid (mg/kg dry mass)						
	0.818* [a]	0.008* [a]	0.461	0.329	0.708	0.044* [a]
p-Coumaric acid (mg/kg dry mass)						
	0.390	0.464	0.219	0.803	0.637	0.094
Ferulic acid (mg/kg dry mass)						
	0.866* [a]	0.002* [a]	0.143	0.919	0.641	0.091

Fig. S6

3.5 Investigations into the Ability to Reduce Cinnamic Acid as Undesired Precursor of Toxicologically Relevant Styrene in Wort by Different Barley to Wheat Ratios (Grain Bill) during Mashing



3.5.1 Summary

So far, barley and wheat varieties were always investigated separately. Interested in whether the phenolic acid contents and enzyme activities between these two grain types might interfere with each other, when mixing them for wheat beer brewing, in this study wort was prepared from different barley to wheat malt ratios and screened for their soluble and free phenolic acid contents.

Thereby, linear correlations were found between the soluble and free cinnamic, *p*-coumaric, and ferulic acid contents and the percentage of wheat malt in the grain bill. In fact, this demonstrated that the phenolic acid contents were a direct function of their concentration in wort of 100% barley or wheat multiplied by the respective percentage of each grain type used for mashing. This further highlighted the absence of any synergistic effects, when mixtures of barley and wheat malts were used for mashing.
Concerning favourable barley to wheat malt ratios leading to a decrease of soluble and free cinnamic acid, the results of the present study indicated that this decision, whether to use higher or lower wheat malt contents for wheat beer brewing, highly depends on the barley and wheat varieties used for mashing and additionally on the malting parameters. Both the variety and the malting parameters were also varied within these mashing experiments. In detail, depending on these two factors, 100% barley and 100% wheat wort of the present investigation alternately showed higher and lower cinnamic acid contents, which, based on the linear correlation, led to either increasing or decreasing cinnamic acid contents with an increasing percentage of wheat malt in the grain bill.
In case of soluble and free *p*-coumaric acid, increasing wheat malt contents clearly lowered the contents in wort, with the variables barley and wheat variety and malting conditions playing a negligible role. This strongly pronounced dependency on the used grain type was a consequence

of the characteristics of barley malts to show very high total *p*-coumaric acid contents, while the *p*-coumaric acid contents in wheat malts were found to be approximately 10 times lower. Finally, soluble ferulic acid showed an increase and free ferulic acid a decrease with increasing wheat malt contents. This was the consequence of the already described higher soluble and lower free ferulic acid contents in wheat wort and the contrary situation in barley wort. With comparable total ferulic acid contents in malts of both grain types, this in turn could be attributed to the higher arabinoxylan degrading enzyme activities in wheat malts, explaining the higher soluble ferulic acid contents in wheat wort, and to higher feruloyl esterase activities in barley malts, explaining the higher free ferulic acid contents in barley wort. Further, the degree of the increase of the ferulic acid content, especially the soluble form, highly depended on the malting parameters, whereas the variety played only a minor role. This was due to the fact that increasing germination temperatures and decreasing aeration rates led to a stronger increase of the soluble ferulic acid contents in wort in case of the wheat variety compared to the two barley varieties, resulting in a stronger difference in the contents between the two grain types.
Furthermore, the impact of wort boiling was investigated, due to the higher temperatures applied. With the absence of relevant differences between unboiled wort and cast wort, wort boiling was proven to have no effect on the soluble and free phenolic acid contents in wort, and thus, temperatures applied within this process step were too low to cause relevant thermal decarboxylation. This observation was in line with a study performed by Samaras et al. [58], showing the absence of relevant amounts of 2M4VP in malts kiln-dried at temperatures <140 °C. Further, considering the high heat transfer capacity of water during wort boiling, a study by Langos et al. [28] also found no relevant differences in the contents of 4VP and 2M4VP between unboiled wort and cast wort.

The individual contributions of V. Kalb and the co-authors' contributions are specified below:

V. Kalb developed the design of the study and conducted the laboratory work. Malt and wort preparation was performed by T. Seewald at the Chair of Brewing Science and Beverage Technology, Technische Universität Berlin. A. Köhler, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, assisted in sample preparation and S. Kaviani-Nejad, Leibniz-Institute for Food System Biology at the Technical University of Munich, was involved in the mass spectrometric determination of phenolic acids via HPLC-MS/MS. V. Kalb statistically analysed and interpreted the data, discussed the results, and created the figures. He developed the story of the paper, wrote the first draft of the manuscript, and revised the paper. T. Hofmann, Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich, coordinated the research project. Prof. Dr. M. Granvogl, Institute of Food Chemistry, University of Hohenheim, was responsible for the conceptualization, project administration, supervision of the research project, and writing reviews and editing.

3.5.2 Publication

Investigations into the Ability to Reduce Cinnamic Acid as Undesired Precursor of Toxicologically Relevant Styrene in Wort by Different Barley to Wheat Ratios (Grain Bill) during Mashing

V. Kalb, T. Seewald, T. Hofmann, and M. Granvogl

Permanent weblink: https://dx.doi.org/10.1021/acs.jafc.1c03018

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Investigations into the Ability to Reduce Cinnamic Acid as Undesired Precursor of Toxicologically Relevant Styrene in Wort by Different Barley to Wheat Ratios (Grain Bill) during Mashing

Valerian Kalb, Torsten Seewald, Thomas Hofmann, and Michael Granvogl*

Cite This: J. Agric. Food Chem. 2021, 69, 9443−9450 Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Styrene is a food-borne toxicant in wheat beer and due to its classification as possibly carcinogenic to humans by the International Agency for Research on Cancer in 2002, mitigation strategies had to be developed. Aiming at understanding the impact of the barley to wheat malt ratio (grain bill) during mashing on the contents of soluble and free (i) cinnamic, (ii) *p*-coumaric, and (iii) ferulic acid, precursors of (i) styrene and the desired vinyl aromatics (ii) 4-vinylphenol and (iii) 2-methoxy-4-vinylphenol in wheat beer, wort was prepared at four different barley to wheat malt ratios of 100:0, 25:75, 50:50, and 0:100 (w/w). Additionally, the malts were produced at different germination temperatures and aeration rates (12/32, 18/35, 18/25, 24/18, and 26/25 (°C; L/min)) to consider these two further parameters as well. Thereby, soluble and free phenolic acid contents in wort showed linear correlations to the percentage of wheat in the grain bill, highlighting the absence of synergistic effects when mixing barley and wheat malts. In contrast, the results described the phenolic acid contents as a function of the concentrations in the respective barley and wheat wort, multiplied by their percentage in the grain bill. However, a clear recommendation for favorable barley to wheat malt ratios leading to a decrease of soluble and free cinnamic acid in wort could not be made, as the contents in the present study proved to be highly dependent on the barley and wheat varieties used during mashing and the parameters applied during malting. This was not the case for *p*-coumaric acid for which a clear decrease of the soluble and free forms was found with increasing wheat malt contents. Differently, the soluble form of ferulic acid increased with an increasing percentage of wheat malt, while the free form decreased. The malting parameters clearly recommended high germination temperatures and low aeration rates when aiming at a reduction of undesired cinnamic acid in wort. Fortunately, soluble and free *p*-coumaric and ferulic acid contents were only slightly affected, indicating that the formation of the characteristic wheat beer aroma might not suffer when applying these favorable conditions for styrene reduction.

KEYWORDS: *styrene, vinyl aromatics, phenolic acids, wheat beer, food-borne toxicant, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), stable isotope dilution analysis (SIDA)*

INTRODUCTION

Since the early 1970s,[1,2] styrene is known to be part of the volatile fraction of wheat beer. However, its toxicological relevance was unattended until 1996, when the World Health Organization (WHO) published a tolerable daily intake (TDI) of 7.7 μg/kg body weight per day.[3] With the classification as possibly carcinogenic to humans (class 2B) by the International Agency for Research on Cancer (IARC)[4] according to studies on rats[5] and mice[6] in 2002, mitigation strategies for styrene in food had to be developed. In wheat beer, styrene is a food-borne toxicant that is formed from the undesired precursor cinnamic acid, which mainly derives from the barley and wheat malts used for wheat beer brewing. Fermentation was proven to be the critical step in styrene formation, as phenolic off-flavor (Pof)-active top-fermenting yeasts were found to be responsible for the decarboxylation of cinnamic acid.[7,8]

While developing mitigation strategies for styrene, two aroma-active vinyl aromatics, namely 4-vinylphenol (4VP) and 2-methoxy-4-vinylphenol (2M4VP), have to be considered in parallel to maintain the typical clove-like and slightly phenolic wheat beer aroma expected by consumers.[9] The structural similarity between the undesired styrene and the desired vinyl aromatics is the result of the formation of all three vinyl aromatics via the same pathway, with *p*-coumaric acid and ferulic acid as the precursors of 4VP and 2M4VP, and highlights the difficulty of the objective of styrene mitigation.

The main sources of phenolic acids in the brewing process are the used barley and wheat malts. Within both grain, the non-starch polysaccharide arabinoxylan contains the majority of the precursors.[10−12] On a molecular level, the phenolic acids link the linear polysaccharide chains of arabinoxylan to a three-dimensional network,[13,14] which is used as a major building block of the cell walls. During germination, part of the malting

Received: May 20, 2021
Revised: July 8, 2021
Accepted: July 12, 2021
Published: August 5, 2021

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

process, different enzymes start to degrade the polysaccharide backbone of the arabinoxylan structure into fragments of different sizes.[15] Additionally, feruloyl esterase directly cleaves off phenolic acids from arabinoxylan. As a consequence of these degradation processes, three different forms of phenolic acids are formed: (i) insoluble ester-bound, (ii) soluble ester-bound, and (iii) free phenolic acids. During mashing, these degradation processes are still running, as the responsible enzymes are still active after kiln-drying.[16] When separating the grist from the wort, only the soluble forms (= sum of soluble ester-bound and free phenolic acids) can be transferred from malt into wort and are, therefore, later on accessible by yeasts. During the subsequent fermentation step, the desired and undesired vinyl aromatics are formed via decarboxylation in the presence of yeasts from the corresponding phenolic acids. According to Daly et al.,[7] bottom-fermenting yeasts lack the ability to decarboxylate phenolic acids, whereas top-fermenting yeasts where found to form an inhomogeneous group of yeasts with either no, a reduced, or a high Pof-activity. The ability to decarboxylate phenolic acids is related to the so-called FDC1 and POF1 genes,[8,17,18] and their activities seem to be the key factor to explain the different decarboxylation abilities. Yeasts typically used for wheat beer brewing are top-fermenting yeasts with a high Pof-activity, whereas for example lager beers are brewed with bottom-fermenting yeasts. This explains why the wheat beer aroma is dominated by the abovementioned desired vinyl aromatics and why styrene plays no major role in lager beers.[19]

During brewing, each processing step has its own possibility to influence the formation of styrene directly or indirectly and is, therefore, a possible target for styrene mitigation. In a previous study,[20] barley and wheat malts were found to differ in their phenolic acid contents. A further study[21] showed that also the pattern of the enzyme activities, directly or indirectly involved in the release of phenolic acids, differed among these two grain types. Therefore, the question arose, to what extent the barley to wheat malt ratio (grain bill) has an impact on the phenolic acid pattern in wort. Schwarz & Methner[22] determined the free cinnamic acid content in wort produced from different ratios of barley to wheat malts and found a decrease of free cinnamic acid with increasing wheat malt contents.

In the present study, the ability of mashing to mitigate styrene in wheat beer via a reduction of the input of its precursor cinnamic acid during manufacturing was investigated. More precisely, the impact of the barley to wheat ratio on the soluble and free phenolic acid contents in wort was analyzed. Thereby, the focus was not only on the free cinnamic acid contents in wort but also on the soluble cinnamic acid contents, as well as on the contents of these different forms of *p*-coumaric and ferulic acid. Additionally, malts produced at different germination temperatures and aeration rates were used for mashing and soluble and free phenolic acid contents were analyzed before and after wort boiling.

MATERIALS AND METHODS

Malt and Wort Preparation. Malting and mashing experiments were kindly performed by the Chair of Brewing Science and Beverage Technology (Technische Universität Berlin, Berlin, Germany). The applicability of the prepared malts and wort for wheat beer brewing was ensured using the standard brewing analyses according to MEBAK.[23] Two industrially relevant barley varieties, Solist (spring barley (sp), two-rowed (2r), Franconia, Germany) and Quench (sp, 2r, Denmark), both harvest year 2017, and one industrial relevant wheat variety, Dacanto (winter wheat, Denmark), harvest year 2016, were included in the sample set. Malts were prepared as following: grain was steeped in a physiological saline solution (0.9%) for 4 h at 16 °C, followed by an air rest for 20 h. In a second cycle, the samples were steeped for another 3 h and an air rest of further 21 h was applied to adjust a steeping degree of 45%. Throughout the whole steeping process, a constant air flow of 25 L/min with a humidity of >80% was used. Next, the grain was germinated at five combinations of different germination temperatures (°C) and aeration rates (L/min) of 12/32, 18/35, 18/25 (in duplicate), 24/18, and 26/25. Subsequently, green malts were withered at a temperature of 50 °C for 16 h, followed by kilning at a temperature of 80 °C for 4 h to a water content of <5%. Finally, the kilned malts were deculmed.

From these malts, wort has been produced according to an industrial mashing procedure. Therefore, preheated water (43 °C; 400 mL) was added to the grist of four different barley to wheat malt ratios of 100:0, 25:75, 50:50, and 0:100 (65 g each). Subsequently, the following temperature program was applied: 0−30 min, 43 °C; 30−37 min, from 43 to 50 °C; 37−47 min, 50 °C; 47−59 min, from 50 to 62 °C; 59−64 min, 62 °C; 64−67 min, from 62 to 65 °C; 67−78 min, 65 °C; 78−84 min, from 65 to 71 °C; 84−119 min, 71 °C; 119−123 min, from 71 to 75 °C; 123−125 min, 75 °C; and 125−128 min, from 75 to 78 °C. Unboiled wort was boiled for 1 h and hopped to 20 IBU (International Bitterness Unit; CO_2-hop extract, 49.7% α-acids). Samples were taken before and after wort boiling to determine the soluble and free phenolic acid contents that were finally normalized to an original gravity of 12%.

Chemicals. The following chemicals were commercially available: acetonitrile and methanol (Baker, Gliwice, Poland), hydrochloric acid (Merck, Darmstadt, Germany), and cinnamic acid, *p*-coumaric acid, ferulic acid, formic acid (FA), and sodium hydroxide (Sigma-Aldrich; Merck). All solvents were of high-performance liquid chromatography (HPLC) gradient grade, and ultrapure water (>MΩ·cm) was from an in-house source.

Stable Isotopically Labeled Internal Standards. The stable isotopically labeled internal standards (ISTD) [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, and [$^{13}C_3$]-ferulic acid were obtained from Sigma-Aldrich.

Quantitation of Soluble and Free Phenolic Acids in Wort by Stable Isotope Dilution Assays (SIDAs) via High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS). For the determination of soluble and free phenolic acids, wort was prepared as recently described[20] and soluble phenolic acids (= sum of soluble ester-bound and free phenolic acids) were determined from alkaline hydrolyzed wort. Therefore, stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added to aliquots of wort (0.5 mL). Next, a sodium hydroxide solution (0.5 mL, 4 mol/L) was added starting the hydrolysis, and the samples were incubated for 2 h at room temperature (RT) under constant shaking (600 rpm; HLC HeizThermoMixer MHL23; Digital Biomedical Imaging Systems, Pforzheim, Germany). Upon completion of the incubation, hydrochloric acid (0.5 mL, 4 mol/L) was added for neutralization, and the samples were thoroughly vortexed, membrane filtered (0.45 μm, Minisart RC, hydrophilic, 15 mm; Sartorius, Göttingen, Germany), and the phenolic acid contents were determined by HPLC-MS/MS.

Free phenolic acid contents were directly accessible from wort after inhibition of enzyme activities to avoid a further release of free acids. Therefore, methanol (0.8 mL), well-known for its enzyme inhibition properties, was added to wort aliquots (0.8 mL), yielding samples containing 50% of methanol. To these mixtures, the stable isotopically labeled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added and the samples were equilibrated for 30 min at RT via thorough shaking (GFL Orbital Shaker 3005; Gesellschaft für Labortechnik, Burgwedel, Germany).

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

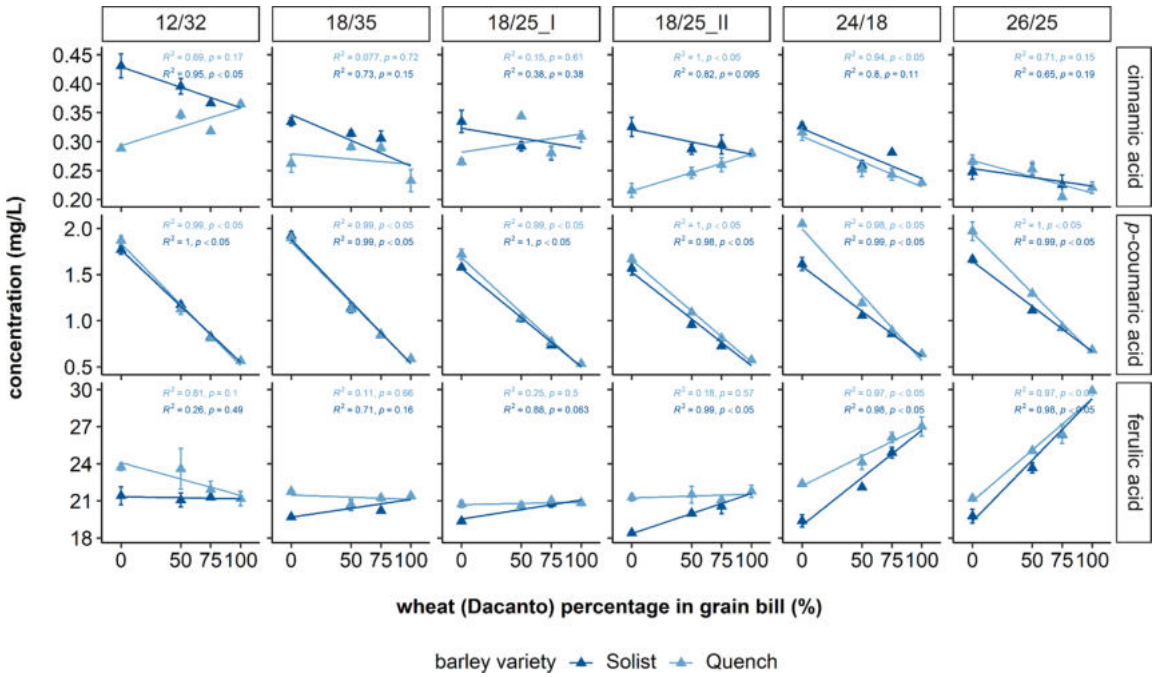


Figure 1. Impact of the barley to wheat malt ratio during mashing (barley varieties: Solist, Quench; wheat variety: Dacanto) on the soluble cinnamic, *p*-coumaric, and ferulic acid contents in wort prepared from malts produced at different germination temperatures (°C) and aeration rates (L/min).

Finally, the samples were membrane filtered (0.45 μm) prior to the quantitation of the free phenolic acid contents via HPLC-MS/MS.

The HPLC-MS/MS consisted of an UltiMate 3000 HPLC system (Thermo Scientific; Dionex Softran, Germering, Germany) coupled to a triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface (TSQ Vantage; ThermoFisher Scientific, Bremen, Germany). Chromatography was performed on a Kinetex C18 column (100 × 2.1 mm, 2.6 μm, 100 Å) equipped with a precolumn of the same type (both Phenomenex, Aschaffenburg, Germany) at 24 °C using a flow gradient, which started at 0.05 mL/min (0−1 min) and linearly increased to 0.2 mL/min (1−2 min) where it was kept constant throughout the rest of the run (2−30 min). The injection volume was generally 20 μL, mobile phase A was 0.1% FA in water (v/v), and mobile phase B was 0.1% FA in acetonitrile (v/v). Gradient elution started with 10% B (0−2 min), linearly increased to 90% B (2−15 min), kept constant at 90% (15−18 min), decreased to 10% B (18−19 min), and kept constant at 10% B (19−30 min) to equilibrate the column. The mass spectrometer was operated with the following parameters in $APCI^+$ mode: vaporizer temperature 250 °C, discharge current 4.0 μA, sheath gas pressure 30 arbitrary units (au), auxiliary gas pressure 10 au, declustering voltage −10 V, and capillary temperature 300 °C. Quantitation of phenolic acids was performed in multiple reaction monitoring (MRM) mode. From the fragment ions, the most intensive one was chosen as the quantifier ion, together with two qualifier ions.[20]

Calibration lines were determined from mixtures of known contents of the unlabeled analyte and the corresponding stable isotopically labeled internal standard in 7 different concentration ratios (10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10) with good linearity (R^2 > 0.99) for all analytes in the applied range.[20]

Statistical Analysis. Means and standard deviations were calculated using Microsoft Office 365 Excel (Microsoft Corporation, Seattle, WA). Data visualization and statistical analysis were performed applying the R statistical software (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria)[24] using the interface R Studio (version 1.1.423; RStudio, Boston, MA).[25] Scatter plots were created using the package ggplot2 (version 3.3.2),[26] regression lines were added using the function geom_smooth (method = lm), and correlation coefficients with *p*-values were calculated using the function stat_cor (method = pearson) from the package ggpubr (version 0.3.0).[27]

RESULTS AND DISCUSSION

Dependency of the Soluble and Free Cinnamic, *p*-Coumaric, and Ferulic Acid Contents in Wort on the Barley to Wheat Malt Ratio Applied during Mashing. Wheat beer brewing has a long tradition in Germany and Austria and its preparation from two different grain types, namely barley and wheat, was even considered in the German purity law. Indeed, a wheat malt content of at least 50% is mandatory in wheat beer production, which is different to most other beer types typically brewed from 100% barley malt. Very recently, it was shown that these two grain types differ in their total (= sum of insoluble ester-bound, soluble ester-bound, and free phenolic acids), soluble (= sum of soluble ester-bound and free phenolic acids), and free cinnamic, *p*-coumaric, and ferulic acid pattern.[20] Additionally, also differences in the enzyme activities, directly or indirectly involved in the release of phenolic acids, were found among barley and wheat varieties.[21] To which extent different ratios of barley to wheat malts during mashing allow to alter the soluble and free cinnamic, *p*-coumaric, and ferulic acid contents in wort and, therefore, might be a possible tool for styrene mitigation, was subject of the present study.

Malts of the two barley varieties Solist and Quench and of the wheat variety Dacanto, prepared at different germination

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

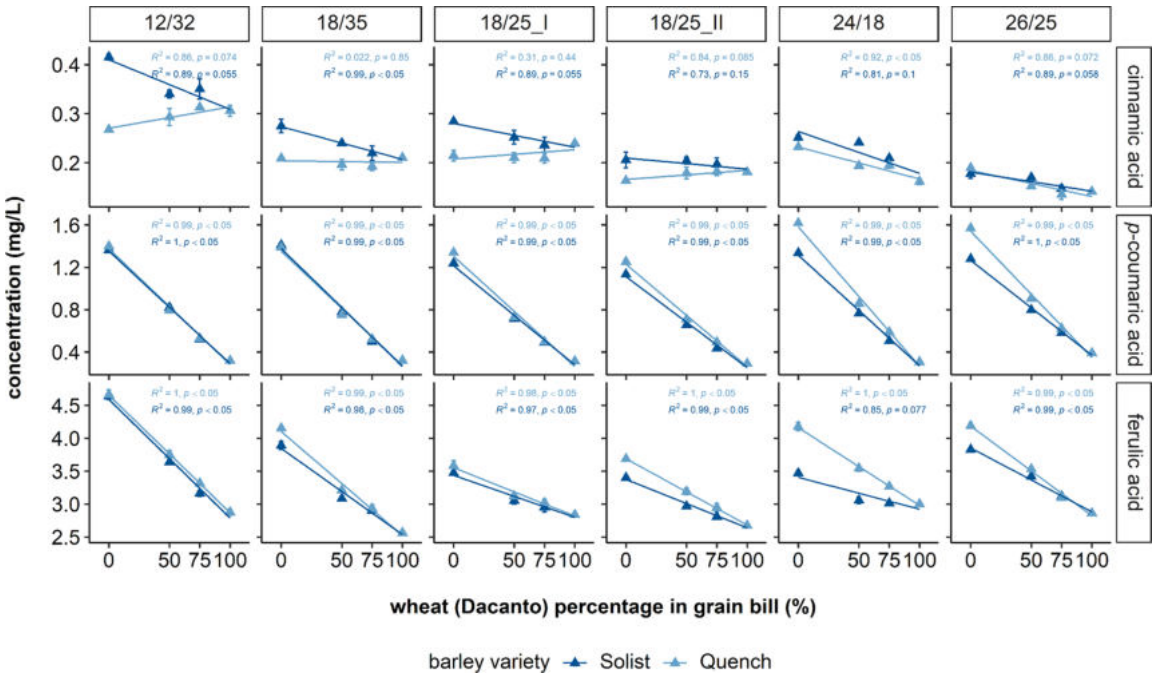


Figure 2. Impact of the barley to wheat malt ratio during mashing (barley varieties: Solist, Quench; wheat variety: Dacanto) on the free cinnamic, *p*-coumaric, and ferulic acid contents in wort prepared from malts produced at different germination temperatures (°C) and aeration rates (L/min).

temperatures and aeration rates, were used for the mashing experiments. Figures 1 and 2 depict the soluble and free desired and undesired phenolic acid contents in wort produced at four different barley to wheat malt ratios containing 0, 50, 75, or 100% of wheat malt. Linear correlations were found between the soluble and free cinnamic (only with some exceptions), *p*-coumaric, and ferulic acid contents and the percentage of wheat malt which means that for every barley to wheat malt ratio, the soluble and free phenolic acid contents in wort can be calculated from their concentrations present in 100% barley and 100% wheat wort, multiplied by the respective percentage of each grain type used for mashing. This finding highlighted that no synergistic effects, for example higher feruloyl esterase activities in barley[21] combined with higher ferulic acid contents in wheat,[20] leading to significantly higher free ferulic acid contents in mixed wort compared to wort prepared from only one grain type, were found, when mixtures of barley and wheat malts were used for mashing.

Wort produced from 100% barley malt of the variety Solist always showed higher amounts of soluble cinnamic acid compared to wort prepared from 100% wheat malt, independent of the malting parameters (Figure 1). Concerning the linear correlation, this had the consequence that increasing amounts of wheat malt in the grain bill of the respective wort led to a decrease (8−30%; max. differences between wort prepared from 100% barley and 100% wheat malt with respect to the different malting parameters) of the soluble cinnamic acid contents. The barley variety Quench showed a less clear picture since in three out of six cases, as a consequence of the malting parameters, the content of soluble cinnamic acid in wort produced from 100% barley malt was lower compared to wort prepared from 100% wheat malt. In those cases, an opposite trend was present, leading to an increase (17−30%) of the soluble cinnamic acid content with an increasing percentage of wheat malt in the grain bill. However, from a statistical point of view, these observations can only be seen as trends and by mitigating the soluble cinnamic acid content in wort via the percentage of wheat malt in the grain bill, malting parameters and the varieties applied for mashing have to be considered as well.

Regarding the amounts of soluble *p*-coumaric acid in wort, a much clearer pattern was found. Independent of the malting parameters and for both barley varieties, a significant decrease of the soluble *p*-coumaric acid contents was found with an increase of the wheat percentage in the grain bill (Figure 1). By comparison of wort produced from 100% barley malt to wort produced from 100% wheat malt, differences in the concentrations of up to 144−232% were found. This strongly pronounced dependency of the amount of soluble *p*-coumaric acid on the used grain type was a consequence of the previously described characteristic of malts of different barley varieties to show very high total *p*-coumaric acid contents (221−419 mg/kg dry mass), whereas in malts of different wheat varieties total *p*-coumaric acid contents were significantly lower (36−40 mg/kg dry mass).[20] This fact was also confirmed by Szwajgier and Borowiec[28] reporting on total *p*-coumaric acid contents of 247 mg/kg dry mass in Pilsner type barley malts but only 53 mg/kg dry mass in pale wheat malts.

Regarding soluble ferulic acid, wort prepared from malts of 100% Solist (barley) always showed lower contents compared to wort produced from 100% wheat malts (Figure 1). Therefore, increasing wheat malt contents within mashing led to an increase of the soluble ferulic acid contents in the respective wort. The degree of this increase was also dependent

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

on the malting parameters, as soluble ferulic acid contents in 100% wheat wort were found to increase significantly with increasing germination temperatures and decreasing aeration rates (Figure 1). As simultaneously the contents in the wort produced from 100% barley malt decreased slightly, the wheat malt content in the grain bill had a much stronger impact on the soluble ferulic acid content at higher germination temperatures and lower aeration rates. With respect to the malting parameters, soluble ferulic acid contents increased by 8−51% (0% for 12/32) if comparing 100% Solist (barley) wort to 100% wheat wort. In case of the barley variety Quench, soluble ferulic acid contents in wort were slightly higher at lower germination temperatures and higher aeration rates compared to the wheat variety, leading to a decrease (2−11%) of soluble ferulic acid at these malting parameters with increasing wheat malt contents. At higher germination temperatures and lower aeration rates, soluble ferulic acid contents in wort prepared from 100% wheat malt exceeded the contents in wort produced from 100% Quench, whereupon again an increase of the wheat malt content during mashing led to an increase of the soluble ferulic acid contents (1−41%) in the respective wort (Figure 1). Comparing these results to a former study[20] that clearly showed higher soluble ferulic acid contents in wort of three wheat varieties (12.8−15.0 mg/L) compared to four barley varieties (8.8−10.7 mg/L), it has to be considered, that soluble ferulic acid contents, and additionally, the degree of the impact of the barley to wheat malt ratio, are not only determined by the grain type, but also by the applied malting parameters.

The same study[20] revealed that in wort of barley and wheat, already >94% and >70% of cinnamic acid and >78% and >77% of *p*-coumaric acid existed in the free form. This fact explains why the pattern of the free cinnamic and *p*-coumaric acid contents in wort matched very well the pattern found for the corresponding soluble forms (cf. Figures 1 and 2; Figure 3). In parallel to the soluble forms, free cinnamic acid contents decreased (12−36%) with increasing wheat malt contents during mashing, when Solist was used as the barley variety. If Solist was replaced by Quench, half of the samples showed a decrease (6−30%) and half of the samples an increase (11−14%) with increasing wheat malt contents in the grain bill. Again, this was a consequence of the malting parameters leading to either slightly higher or lower contents in wort produced from Quench compared to wort produced from the wheat variety. Overall, these results were consistent with a study by Schwarz and Methner,[22] in which also a decrease of free cinnamic acid in wort was found with increasing wheat malt contents. The presence of high standard deviations, as a consequence of the different barley and wheat varieties and different malting conditions, highlighted again the influence of these factors on the phenolic acid contents. Regarding free *p*-coumaric acid in the present study, again a massive decrease (70−81%) was found with increasing wheat malt contents, again as the result of the characteristic low total amounts in wheat malts compared to barley malts.[20]

For free ferulic acid, a different pattern was found (Figure 2). In opposite to the increase of the soluble form, a clear decrease (14−38% lower if comparing 100% barley (Quench and Solist) wort to 100% wheat wort) was found with increasing wheat malt contents (cf. Figures 1 and 2; Figure 3). In accordance with the results of a former study,[29] the higher soluble and lower free contents in wheat wort and the contrary situation in barley wort was responsible for the increase of

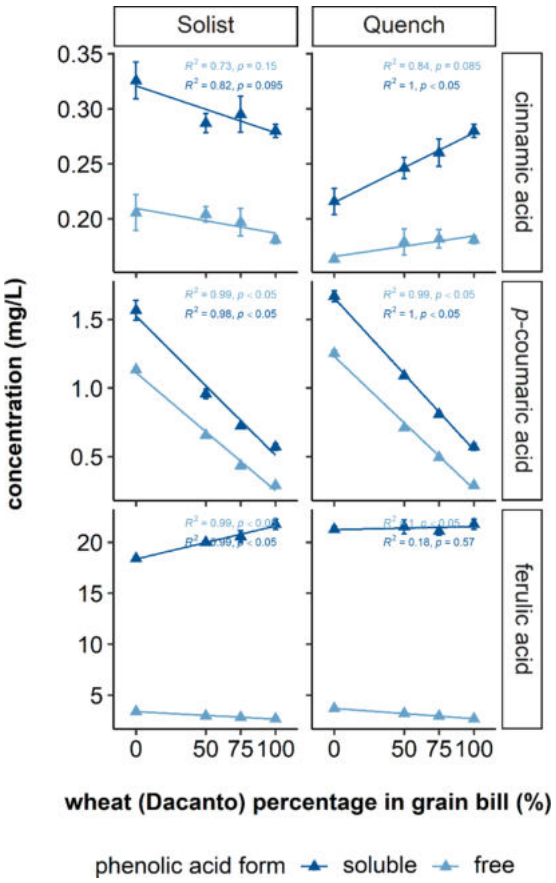


Figure 3. Comparison of the dependence of the soluble and free cinnamic, *p*-coumaric, and ferulic acid contents in wort (barley varieties: Solist, Quench; wheat variety: Dacanto) on the barley to wheat malt ratio. Representative data from wort produced from malts germinated at 18 °C and an aeration rate of 25 L/min.

soluble ferulic acid and the decrease of free ferulic acid with increasing wheat malt contents in the grain bill. With comparable amounts of total ferulic acid in malts of barley (1490−1930 mg/kg dry mass) and wheat (1690−1800 mg/kg dry mass) shown by previous studies,[20,29] the enzyme activities present in these grain types might be the key role in explaining the higher soluble ferulic acid contents in wheat wort compared to the higher free ferulic acid contents in barley wort.

In a previous study,[21] water-extractable arabinoxylan contents were found to be significantly higher in wheat malts (0.59−0.79 g/100 g dry mass) compared to barley malts (0.93−1.51 g/100 g dry mass), suggesting that arabinoxylan degrading enzymes play a more important role in wheat, where they will lead to a stronger formation of soluble ester-bound ferulic acids. Contrary, in recent studies,[21,29] feruloyl esterase activities were found to be significantly higher in barley malts (10.8−14.7 U/g dry mass) compared to wheat malts (0.48−8.3 U/g dry mass). The higher contents of free ferulic acid in the present barley wort can be explained by the fact that the feruloyl esterase is still active after kiln-drying.

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

Journal of Agricultural and Food Chemistry pubs.acs.org/JAFC Article

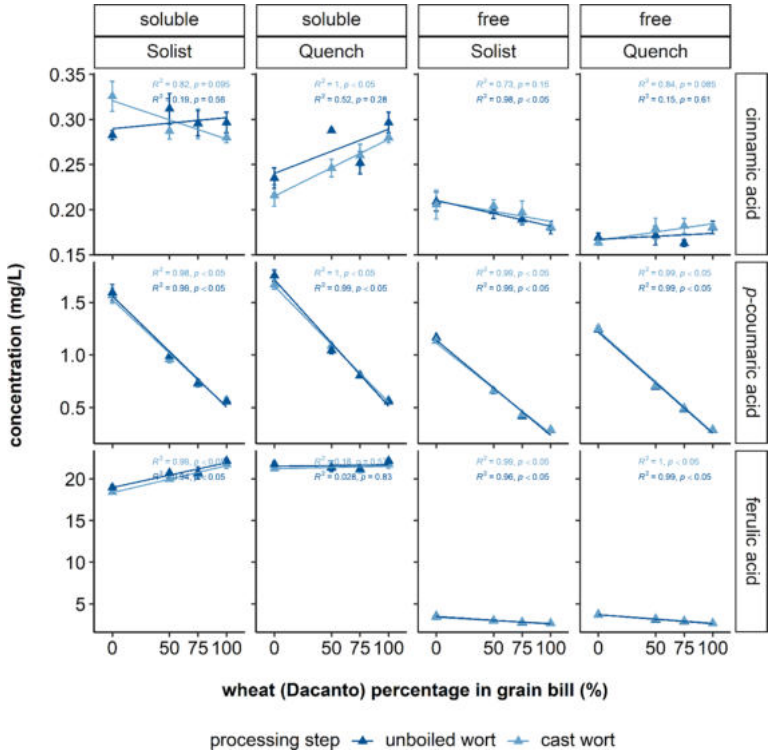


Figure 4. Influence of wort boiling on the soluble and free cinnamic, *p*-coumaric, and ferulic acid contents in wort produced from different barley to wheat malt ratios (barley varieties: Solist, Quench; wheat variety: Dacanto). Representative data from wort produced from malts germinated at 18 °C and an aeration rate of 25 L/min.

Influence of Wort Boiling on the Soluble and Free Cinnamic, *p*-Coumaric, and Ferulic Acid Contents in Wort. During wort boiling, various important processes occur, for example, evaporation of undesirable aroma compounds, destruction of all enzymes, wort sterilization, extraction of hop components, and formation and precipitation of protein-polyphenol adducts.[30] Aiming at evaluating the impact of this processing step, which is mainly related due to the high temperature applied, samples were taken before and after wort boiling. Figure 4 exemplarily compares the contents of the soluble and free forms of cinnamic, *p*-coumaric, and ferulic acid in unboiled wort and cast wort prepared from malts germinated at 18 °C with an aeration rate of 25 L/min. With mean differences of 8% and 4% of the soluble and free cinnamic acid contents and 3% and 2% of the soluble and free *p*-coumaric and ferulic acid contents between cast wort and unboiled wort, no relevant impact on the contents of these analytes was found by wort boiling. This finding fits to the observation made by Samaras et al.[31] analyzing the amounts of free *p*-coumaric and ferulic acid as a function of the roasting degree of barley malts. In malts kiln-dried at >220 °C, they found a significant reduction of free phenolic acids together with a clear increase of the concentration of 2M4VP up to 267−439 μg/kg dry mass. In contrast, malts kiln-dried at temperatures <140 °C revealed the absence of relevant amounts of 2M4VP (not detectable), indicating that thermal decarboxylation processes at temperatures <140 °C played a negligible role. Further, it had to be considered that kilning is a dry heating process, whereas wort boiling is a wet heating process with a temperature of 100 °C. However, this concern was addressed by a study of Langos and Granvogl,[32] showing only small differences in the contents of 4VP and 2M4VP in unboiled wort and cast wort, also proving that during wort boiling, thermal decarboxylation processes do not contribute to the formation of 4VP and 2M4VP in wheat beer to a relevant extent.

Impact of the Germination Temperature and Aeration Rate during Malting on the Soluble and Free Cinnamic, *p*-Coumaric, and Ferulic Acid Contents in Wort. Additionally to the variation of the barley to wheat malt ratio during mashing, malts were prepared at five different combinations of germination temperatures and aeration rates (12/32, 18/35, 18/25 (standard parameters typically used for malting), 24/18, and 26/25 (°C; L/min)). In fact, these were the most interesting parameters of a response surface approach of a former study,[29] which showed the greatest differences among total, soluble, and free phenolic acid contents in malts. For both 100% barley and 100% wheat wort of the present study, soluble cinnamic acid contents decreased with increasing germination temperatures and decreasing aeration rates (Figure 1). This decrease was even stronger for free cinnamic acid (Figure 2). As wort of the barley and wheat varieties were similarly affected, this led to an overall decrease of soluble and free cinnamic acid contents at each barley to wheat malt ratio (cf. Figures 1 and 2). Therefore, higher germination temperatures and lower aeration rates favor

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

styrene mitigation. In comparison, the impact on *p*-coumaric acid was rather small for both barley and wheat wort. Soluble and free *p*-coumaric acid contents were slightly higher at the extremes (12/32, 18/35, 24/18, and 26/25) compared to the standard malting parameters (18/25; cf. Figures 1 and 2). This finding is very important and helpful concerning styrene mitigation, as the desired precursors have to be maintained in sufficient amounts, to ensure the formation of the characteristic wheat beer aroma expected by consumers.

Regarding soluble ferulic acid, wort of the barley varieties had slightly higher contents at a germination temperature of 12 °C and an aeration rate of 32 L/min compared to the other parameter combinations, among whom no further relevant changes were found. This was different in wheat wort where soluble ferulic acid contents formed a plateau between the malting parameter combinations of 12/32 and 18/25, followed by a strong increase caused by the malting parameter combinations of 24/18 and 26/25 (Figure 1). This increase of soluble ferulic acid in wheat was assumed to be the consequence of an increase of the activity of arabinoxylan degrading enzymes triggered by the higher germination temperatures,[15] which interestingly seemed to play no role in the barley varieties. In contrast, free ferulic acid was differently affected by the malting parameters. In case of the barley varieties Solist and Quench, a minimum was formed at a germination temperature of 18 °C and an aeration rate of 25 L/min, with higher free ferulic acid contents at the extremes. However, free ferulic acid contents in 100% wheat wort were unaffected by the malting parameters (Figure 2). Therefore, favorable malting parameters for styrene mitigation will not affect the soluble and free ferulic acid contents to a degree which would prevent the formation of the characteristic wheat beer aroma.

In summary, linear correlations between soluble and free phenolic acid contents in wort and the percentage of wheat in the grain bill used for mashing revealed that these contents are dependent on the barley to wheat ratios in the grain bill. Also, no synergistic effects caused, for example, by higher feruloyl esterase activities in barley combined with higher ferulic acid contents in wheat, were found by combining these two grain types. Further, the present results indicated that malting at high germination temperatures and low aeration rates reduced soluble and free cinnamic acid in wort, and thus, the styrene content in the final beer. The decision which percentage of wheat malt in the grain bill is favorable for a decrease of soluble and free cinnamic acid in wort highly depends on the barley and wheat varieties used for mashing. This was different for soluble and free *p*-coumaric acid present at clearly lower amounts in wort with increasing wheat malt contents, while ferulic acid showed a decrease for the free form and an increase for the soluble form. In general, wort boiling had no effect on the soluble and free phenolic acid contents in wort, as temperatures applied during this process step were too low for a thermal decarboxylation. Finally, these soluble and free cinnamic acid mitigation measures seem to minimally affect the contents of soluble and free *p*-coumaric and ferulic acid, allowing the maintenance of the characteristic wheat beer aroma expected by the consumers in parallel with a mitigated content of the undesired styrene.

AUTHOR INFORMATION

Corresponding Author

Michael Granvogl − *Fachgebiet Lebensmittelchemie und Analytische Chemie (170a), Institut für Lebensmittelchemie, Fakultät Naturwissenschaften, Universität Hohenheim, D-70599 Stuttgart, Germany*; orcid.org/0000-0003-1281-9999; Phone: +49 711 459 23979; Email: michael.granvogl@uni-hohenheim.de; Fax: +49 711 459 24096

Authors

Valerian Kalb − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany*

Torsten Seewald − *Institut für Lebensmitteltechnologie und Lebensmittelchemie, Fachgebiet Brauwesen, Technische Universität Berlin, D-13353 Berlin, Germany*

Thomas Hofmann − *Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, D-85354 Freising, Germany; Leibniz-Institut für Lebensmittel-Systembiologie an der Technischen Universität München, D-85354 Freising, Germany;* orcid.org/0000-0003-4057-7165

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c03018

Funding

The IGF Project (AiF 19593 N) of the Association for the Promotion of Science of the German Brewing Industry (Wifö) was supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a decision by the German Bundestag.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We express many thanks to Miss Anneliese Köhler for technical assistance and Mr. Sami Kaviani-Nejad for performing the LC-MS/MS measurements.

REFERENCES

(1) Renner, R.; Bartels, H.; Tressl, R. Enrichment and quantitative determination of beer aroma substances by gas extraction (in German). *Monatsschr. Brau.* **1976**, *29*, 478−480.

(2) Tressl, R.; Friese, L.; Fendesack, F.; Koeppler, H. Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. *J. Agric. Food Chem.* **1978**, *6*, 1422−1426.

(3) WHO. *Guidelines for Drinking-Water Quality: Health Criteria and Other Supporting Information*, 2nd ed.; World Health Organization: Geneva, Switzerland, 1996.

(4) IARC. *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, some Mycotoxins, Naphtalene and Styrene*; International Agency for Research on Cancer: Lyon, France, 2002.

(5) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicol. Sci.* **1998**, *46*, 266−281.

(6) Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Bevan, C.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in

CD-1 mice by inhalation exposure for 104 weeks. *J. Appl. Toxicol.* **2001**, *21*, 185−198.
(7) Daly, B.; Collins, F.; Madigan, D.; Donelly, D.; Coakley, M.; Ross, P. An investigation into styrene in beer. *Proc. Congr. - Eur. Brew. Conv.* **1997**, *26*, 623−630.
(8) Goodey, A. R.; Tubb, R. S. Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *J. Gen. Microbiol.* **1982**, *128*, 2615−2620.
(9) Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. *J. Agric. Food Chem.* **2013**, *47*, 11303−11311.
(10) Dvořáková, M.; Guido, L. F.; Dostálek, P.; Skulilová, Z.; Moreira, M. M.; Barros, A. A. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *J. Inst. Brew.* **2008**, *1*, 27−33.
(11) Nordkvist, E.; Salomonsson, A.-C.; Åman, P. Distribution of insoluble bound phenolic acids in barley grain. *J. Sci. Food Agric.* **1984**, *35*, 657−661.
(12) Maillard, M.-N.; Berset, C. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. *J. Agric. Food Chem.* **1995**, *7*, 1789−1793.
(13) Gubler, F.; Ashford, A. E. Release of ferulic acid esters from barley aleurone. I. Time course of gibberellic-acid-induced release from isolated layers. *Aust. J. Plant Physiol.* **1985**, *3*, 297−305.
(14) Fincher, G. B. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 305−346.
(15) Rasmussen, C. V.; Boskov Hansen, H.; Hansen, Å.; Melchior Larsen, L. pH-, temperature- and time-dependent activities of endogenous endo-β-D-xylanase, β-D-xylosidase and α-L-arabinofuranosidase in extracts from ungerminated rye (*Secale cereale* L.) grain. *J. Cereal Sci.* **2001**, *1*, 49−60.
(16) Humberstone, F. J.; Briggs, D. E. Extraction and assay of ferulic acid esterase from malted barley. *J. Inst. Brew.* **2000**, *1*, 21−29.
(17) Mukai, N.; Masaki, K.; Fujii, T.; Kawamukai, M.; Iefuji, H. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* **2010**, *6*, 564−569.
(18) Payne, K. A. P.; White, M. D.; Fisher, K.; Khara, B.; Bailey, S. S.; Parker, D.; Rattray, N. J. W.; Trivedi, D. K.; Goodacre, R.; Beveridge, R.; Barran, P.; Rigby, S. E. J.; Scrutton, N. S.; Hay, S.; Leys, D. New cofactor supports α,β-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition. *Nature* **2015**, *7557*, 497−501.
(19) Kalb, V.; Granvogl, M. Formation of Desired Smoky Key Odorants in Wheat Beer: a Comparison with the Undesired Toxicologically Relevant Styrene. In *Sex, Smoke, and Spirits: The Role of Chemistry*; ACS Symposium Series 1321; Guthrie, B., Beauchamp, J. D., Buettner, A., Toth, S., Qian, M. C., Eds.; American Chemical Society: Washington, DC, 2019; pp 93−105.
(20) Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Studies on the impact of malting and mashing on the free, soluble ester-bound, and insolbule ester-bound forms of desired and undesired phenolic acids aiming at styrene mitigation during wheat beer brewing. *J. Agric. Food Chem.* **2020**, *68*, 12421−12432.
(21) Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The role of endogeneous enzymes during malting of barley and wheat varieties in the mitigation of styrene in wheat beer. *J. Agric. Food Chem.* **2020**, *68*, 13888−13896.
(22) Schwarz, K. J.; Methner, F.-J. Styrene concentrations during wheat beer production. *BrSc.* **2011**, *64*, 156−158.
(23) Pfenninger, H. *Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK)*, 3rd ed.; Brautechnische Analysenmethoden 1; Selbstverlag der MEBAK: Freising, Germany, 1997.
(24) R Development Core Team. *A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2019; http://www.r-project.org/ (accessed 2020/4/14).
(25) RStudio Team. *RStudio: Integrated Development for R*. RStudio: Boston, MA, 2019; http://www.rstudio.com/ (accessed 2020/4/14).
(26) Wickham, H.; Chang, W.; Henry, L.; Pedersen, T. L.; Takahashi, K.; Wilke, C.; Woo, K.; Yutani, H.; Dunnington, D. *R ggplot2: Elegant Graphics for Data Analysis*, 2020; https://cran.r-project.org/package=ggplot2 (accessed 2020/11/17).
(27) Kassambra, A. *R ggpubr: Based Publication Ready Plots*, 2020; https://cran.r-project.org/package=ggpubr (accessed 2020/11/17).
(28) Szwajgier, D.; Borowiec, K. Phenolic acids from malt are efficient acetylcholinesterase and butyrylcholinesterase inhibitors. *J. Inst. Brew.* **2012**, *1*, 40−48.
(29) Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The malting parameters steeping temperature, germination temperature, aeration rate, and withering temperature as possibilities for styrene mitigation in wheat beer. *Eur. Food Res. Technol.* **2021**, submitted.
(30) Kunze, W. *Technology Brewing and Malting*, 5th ed.; Versuchs- und Lehranstalt für Brauerei: Berlin, Germany, 2016.
(31) Samaras, T. S.; Camburn, P. A.; Chandra, S. X.; Gordon, M. H.; Ames, J. M. Antioxidant properties of kilned and roasted malts. *J. Agric. Food Chem.* **2005**, *20*, 8068−8074.
(32) Langos, D.; Granvogl, M. Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. *J. Agric. Food Chem.* **2016**, *11*, 2325−2332.

https://doi.org/10.1021/acs.jafc.1c03018
J. Agric. Food Chem. 2021, 69, 9443−9450

4 Further Experiments

4.1 Structure Elucidation of Soluble Ester-Bound Phenolic Acids in Malt

4.1.1 Objective

Soluble ester-bound phenolic acids are thought to play an important role in understanding the coherence between the phenolic acid contents in one of the process intermediates and the vinyl aromatic contents in wheat beer. In fact, this is related to its soluble nature, allowing phenolic acids, besides the free form, to be transferred from malts into wort during mashing. Therefore, with the feruloyl esterase being still active after kiln-drying [75] and the presence of the yeast's own feruloyl esterase [76], the soluble forms are an additional source of phenolic acids for the decarboxylation process. Aiming at the structure elucidation of soluble ester-bound phenolic acids, hot water extracts were analysed by means of ultra high-performance liquid chromatography-time-of-flight-mass spectrometry (UHPLC-ToF-MS) using the technique of Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS) [83]. From these measurements, data sets were obtained with an fragmentation spectrum for every eluting substance, which were the basis for the determination of the molecular masses of the fragments. Further, taking the MS-fragmentation rules of polysaccharides [84] into account, their structure can successfully be elucidated.

4.1.2 Materials and Methods

Extraction of Soluble Ester-Bound Phenolic Acids from Malt

For the structure elucidation of soluble ester-bound phenolic acids, malts (0.2 g) were extracted with boiling water (2 mL, 95 ℃) for 1 h at 100 ℃ under constant shaking (600 rpm; HLC HeizThermoMixer MHL23). Beside the extraction of the target analytes, this should lead to the inactivation of enzymes involved in degradation processes. Following, the samples were centrifuged (4,700 rpm, 10 min, RT; Heraeus Multifuge X3 FR) and membrane filtrated (0.45 μm, Minisart RC, hydrophilic, 15 mm). The obtained extracts were diluted 1 + 1 (v + v) with methanol for stabilisation and further subjected to UHPLC-SWATH-MS measurements.

Ultra High-Performance Liquid Chromatography-Time-of-Flight-Mass Spectrometry (UHPLC-ToF-MS)

UHPLC-ToF-MS measurements were acquired on a Shimadzu Nexera X2 system (Kyoto, Japan) coupled to a Sciex TripleToF 6600 mass spectrometer (Darmstadt, Germany). Instrumental control and data aquisition was performed with AnalystTF software (Version 1.7.1; Sciex). Chromatographic separation was accomplished using a Kinetex Phenyl-Hexyl column (100 × 2.1 mm, 1.7 μm, 100 Å) equipped with a guard column of the same type (both Phenomenex, Aschaffenburg, Germany). Thereby, the following conditions were applied: mobile phase A, aqueous formic acid (FA) (0.1% (v/v)), mobile phase B, FA in acetonitrile (0.1% (v/v)); gradient: 0–0.5 min, 5% B; 0.5–14 min, from 5 to 40% B; 14–15 min, from 40 to 100% B; 15–16 min, 100% B; 16–17%, from 100 to 5% B; 17–20 min, 5% B; flow rate: 0.3 mL/min; injection volume: 10 μL; and column temperature: 40 °C. The ion source was operated in the negative electro spray ionisation (ESI^-) mode using the following parameters: ion spray voltage: 4500 eV; source temperature: 550 °C; nebulising gas: 55 psi; heating gas: 65 psi; and curtain gas (N_2): 35 psi.

Data Independent Aquisition (DIA)

In SWATH mode, ToF-MS scans were aquired in the *m/z*-range of 100–1,500 using constant Q1 isolation windows with a width of 75 Da and an overlap of 1 Da. Product ion spectra were accumulated in high resolution mode for 60 ms using a declustering potential of –80 V, a collision energy of –10 V, and a collision energy spread of 15 V. Data processing was performed using the MS-DIAL software (Version 3.98; RIKEN Center for sustainable Resource Science, Yokohama, Japan).

4.1.3 Results and Discussion

Differing from other MS-methods, the SWATH-MS technique provides data sets with a fragmentation spectrum for every possible eluting substance, which in turn can be used for the structure elucidation of unknown compounds. In the concrete case of soluble ester-bound phenolic acids, first, this data set had to be reduced to fragmentation spectra possibly originating from arabinoxylan fragments containing phenolic acids. This was achieved by taking advantage of the fact that every possible fragment, apart from phenolic acid residues, solely consists of arabinose (150.13 g/mol) and xylose (150.13 g/mol), and therefore, must contain at least one mass loss resulting from the cleavage of one sugar unit of *m/z* of 132 (150 – 18, water loss) in its fragmentation spectrum. In the next step, the reduced data set was manually compared to a list of calculated masses of possible soluble ester-bound phenolic acids. For that purpose, basic building blocks, consisting of 1, 2, or 3 phenolic acids as well as from bridging-units (*di*-phenolic acids), were continuously extended by sugar units and the mass of each fragment was calculated. With this approach, the free forms of sinapinic, ferulic, caffeic, *p*-coumaric, and cinnamic acid were found. However, due to low concentrations, only for ferulic acid, additional calculated fragments with *m/z* of 589, 721, 853, and 1,117 could be found. To ensure that these masses

stem from soluble ester-bound phenolic acids, the corresponding fragmentation spectra were inspected for characteristic fragmentations (Figure 4.1).

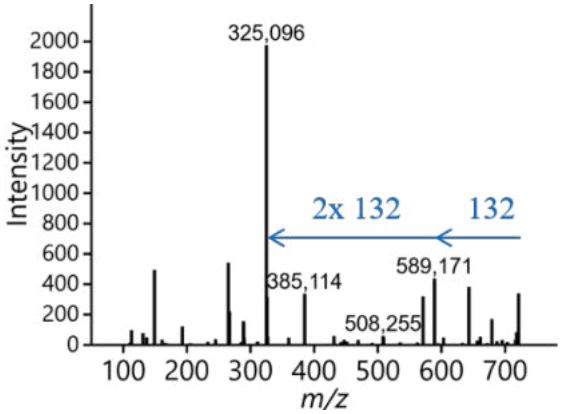


Figure 4.1: MS-degradation spectrum exemplarily shown for the soluble ester-bound phenolic acid with *m/z* 721. Each mass loss of *m/z* 132 corresponds to the loss of one sugar moiety of either xylose or arabinose. (Intensity in cps)

Taking the fragmentation spectra for structure elucidation, each mass loss of 132 contributes to one sugar unit and *m/z* of 325 represents an arabinose unit coupled to one molecule of ferulic acid. In this way, 3, 4, 5, or 7 sugar molecules could be assigned to the four fragments. Thereby, at least one of these sugars had to be an arabinose, as phenolic acids are solely bound via arabinose to the xylose chain (cf. Section 1.4.2). A further assignment, however, was not possible, due to the same molecular weight of arabinose and xylose. Still it could be assumed, that each mass was comprised by an uniformed structure, since the found masses could be matched with only one retention time. Instead, fragments with the same mass, but with different arabinose to xylose ratios, and therefore, different spacial arrangements, should have led to different retention times. Luckily, oligosaccharides follow a distinct fragmentation pattern in mass spectrometry [84]. This fact enabled the final structure elucidation of the found soluble ester-bound phenolic acids. Figure 4.2 exemplarily shows the application of these rules for the structure elucidation of the fragment with the mass of 721 Da. The smallest of these fragments with *m/z* of 589 was composed of two xylose units, to which a ferulated arabinosyl unit was attached at the 3′-position at the reducing end. With an increasing mass of the fragments, this structure was elongated at the reducing end by further xylose units. A comparison to literature [84–86] confirmed the found fragment masses and postulated structures, which were described as relatively short xylose chains with one ferulated arabinosyl unit. Based on the intensities of the respective fragments, a concentration ratio of 7:4:2:1 was determined with an increasing molecular weight. Therefore, the fragment with *m/z* 589 was the most common one, followed by the fragments with *m/z* 721, 853, and 1,117. This suggests an enzymatic fragmentation of arabinoxylan, which favours the formation of small fragments - either by enzymes, only capable for the release of short fragments or by a combination of enzymes, first cleaving the arabinoxylan into still insoluble fragments, which are further degraded from their endings.

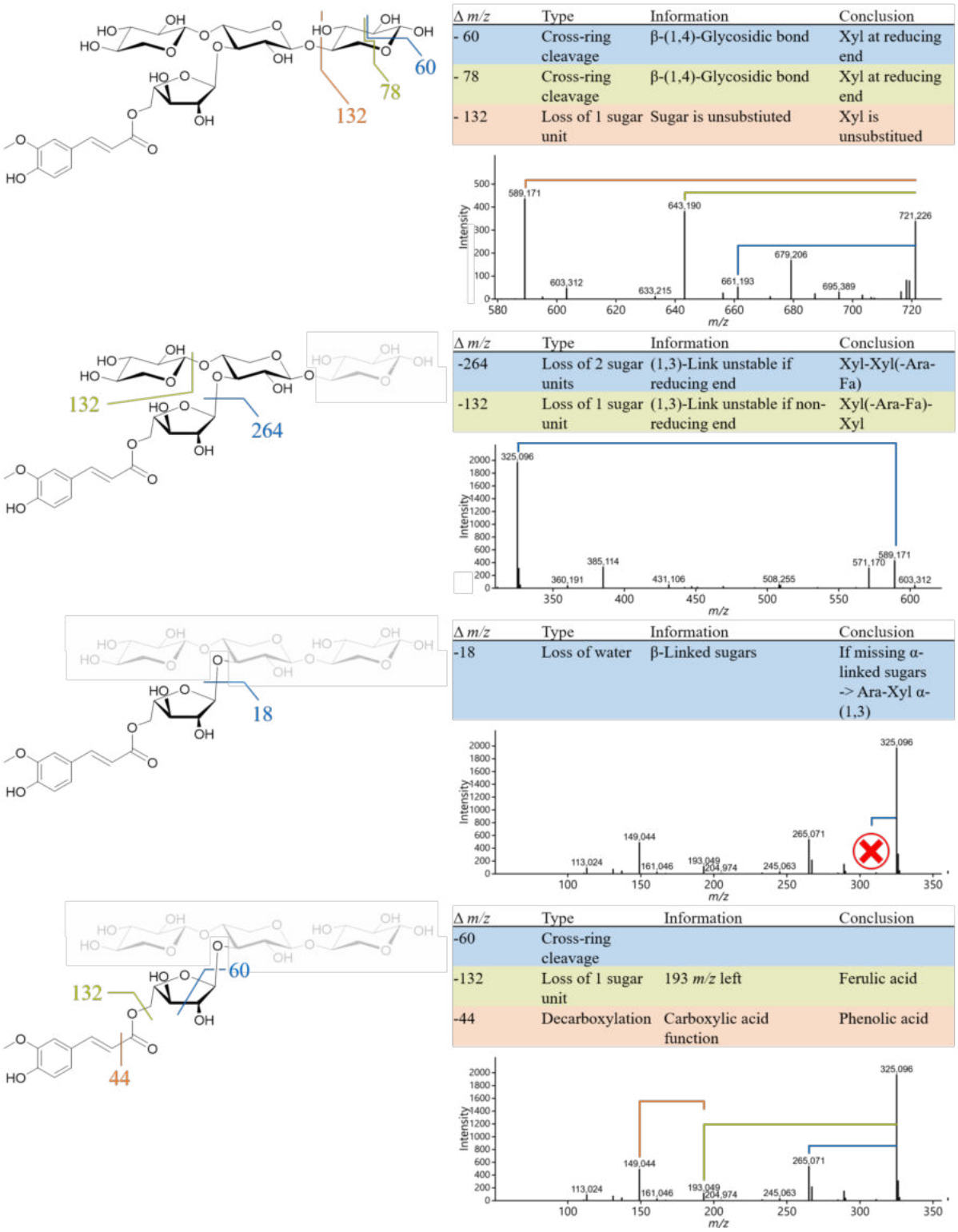


Figure 4.2: Use of the MS-fragmentation rules of polysaccharides for the structure elucidation of soluble ester-bound phenolic acid fragments. The illustration exemplarily shows the structure elucidation of the fragment *m/z* 721. (Xyl, xylose; Ara, arabinose; Fa, ferulic acid; Intensity in cps)

4.2 Fermentation Experiments of Wort Produced from Different Barley to Wheat Malt Ratios Aiming at Unravelling the Correlation between the Contents of the Precursors in Process Intermediates and the Final Vinyl Aromatic Contents in Wheat Beer

4.2.1 Objective

It is already an undisputed fact that the desired and undesired vinyl aromatics in wheat beer are predominantly formed from phenolic acids during fermentation via decarboxylation in presence of top-fermenting yeasts. The more interesting it was that no correlation was found between the free precursor concentrations in the processing steps and the final concentrations of the corresponding vinyl aromatics in wheat beer in former studies [28, 29]. Retrospectively, based on the fact that the feruloyl esterase is still active after kiln-drying [75] and that yeasts have their own feruloyl esterase activity [76], this was not surprising. A further detail which was not included to understand the correlation between phenolic acid contents in process intermediates and final vinyl aromatic concentrations in wheat beer, was the presence of huge amounts of soluble ester-bound phenolic acids in each of these process intermediates.
Aiming at closing this knowledge gap on the role of soluble ester-bound phenolic acids and with the attempt to finally unravel the correlation between the phenolic acid contents in process intermediates and the vinyl aromatic contents in wheat beer, wort samples of section 3.5.2 were fermented in lab-scale and the free and soluble ester-bound phenolic acid contents, as well as the vinyl aromatic concentrations, were determined in the respective wheat beer samples. To study the impact of the percentage of wheat malt in the grain bill on the final vinyl aromatic contents, wort was prepared at different barley to wheat malt ratios.

4.2.2 Materials and Methods

Lab-Scale Fermentation of Wheat Beer Samples

Fermentation experiments were kindly performed by the Chair of Brewing Science and Beverage Technology (Technische Universität Berlin, Berlin, Germany). Wort samples of section 3.5.2 were fermented as following: aliquots (5 mL) of each wort were transferred twice into 50 mL polypropylene (PP)-vials and top-fermenting yeasts of the strain W68 (standard wheat beer yeast) and W127 were added (pitching rate $1.5 * 10^7$ cells/mL). Subsequently, the vials were sealed with a fermentation lock and the samples were fermented for 4 days at 20 °C. Afterwards, sedimented yeast was removed via decanting and the wheat beer samples were frozen to –18 °C prior to analysis. As control, a commercially available wheat beer (unfiltered, pale wheat beer; alcohol content 5.4%; original gravity 12.4%) was included into the sample set.

Chemicals

The following chemicals were commercially available: methyltriphenylphosphonium bromide (Alfa Aesar, Haverhill, MA); acetonitrile (Baker, Gliwice, Poland); styrene (Fluka, Neu-Ulm, Germany); dichloromethane, ethanol, ethyl acetate, *n*-hexane, hydrochloric acid, sodium chloride, and anhydrous sodium sulfate (Merck, Darmstadt); cinnamic acid, *p*-coumaric acid, potassium *tert*-butanolate, ferulic acid, formic acid, 2-methoxy-4-vinylphenol, sodium hydroxide, tetrahydrofurane (THF), and 4-vinylphenol (Sigma-Aldrich; Merck); [$^2H_5$]-4-hydroxy-benzaldehyd (Toronto Research Chemicals, Toronto, Canada); and argon, helium, hydrogen, and nitrogen (Westfalen, Münster, Germany). All solvents were of high-performance liquid chromatography (HPLC) gradient grade, and ultrapure water was from an in-house source.

Stable Isotopically Labelled Internal Standards

The following stable isotopically labelled internal standards were commercially obtained: [$^2H_7$]-cinnamic acid, [$^{13}C_3$]-*p*-coumaric acid, [$^{13}C_3$]-ferulic acid, and [$^2H_8$]-styrene (Sigma-Aldrich); and [$^2H_3$]-2-methoxy-4-vinylphenol (Toronto Research Chemicals, North York, ON, Canada). [$^2H_5$]-4-Vinylphenol was synthesised according to a newly developed method (see below).

Synthesis of [$^2H_5$]-4-Vinylphenol

A new synthesis strategy was developed for the preparation of stable isotopically labelled 4-vinylphenol, which was based on a Wittig-reaction motivated by the work of Farina et al. [87], Albert et al. [88], and Garcia-Barrantes et al. [89].

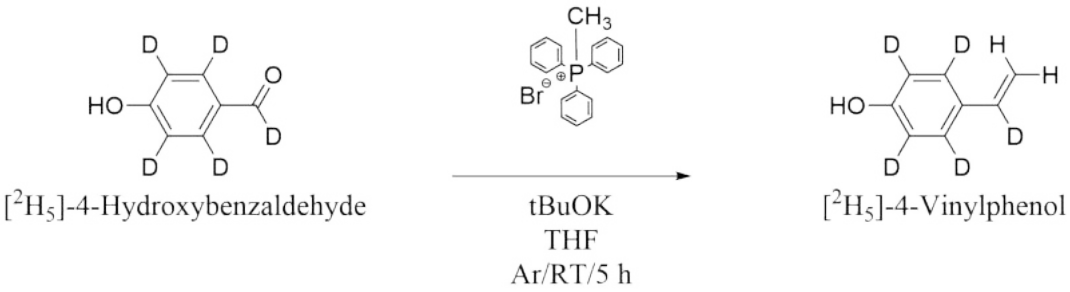


Figure 4.3: Synthesis of [$^2H_5$]-4-vinylphenol. One-step synthesis of [$^2H_5$]-4-vinylphenol via Wittig-reaction starting from [$^2H_5$]-4-hydroxybenzaldehyde.

The synthesis was carried out under inert atmosphere (Argon). First, methyltriphenylphosphonium bromide (1861 mg, 5.21 mmol, 2.6 eq.) was transferred into a three-necked flask and suspended subsequently in THF (10 mL). To this suspension, potassium *tert*-butanolate (632 mg, 5.63 mmol, 2.9 eq.) was added stepwise, and the mixture was stirred for one hour at room temperature (RT). Next, [$^2H_5$]-4-hydroxybenzaldehyde (250 mg, 1.97 mmol, 1 eq.) was dissolved in THF (5 mL) and added dropwise (1 drop per 10 sec). After addition, the reaction mixture was further stirred at RT. A complete conversion of the educt was confirmed after 5 h via thin-layer chomatography (silica gel 60 F254; Merck; *n*-hexane + ethyl acetate =

5 + 1, v + v). Subsequent, dichloromethane (25 mL) was added and the organic phase was washed with deionized water (2 x 20 mL) and a saturated sodium chloride solution (2 x 20 mL) to remove the excess of methyltriphenylphosphonium bromide and potassium *tert*-butanolate. The organic phase was then dried over anhydrous sodium sulfate, filtered (paper filter, 5 H/N, 240 mm, 85 g/m^2; Sartorius, Göttingen, Germany), and the solvent was removed via a rotary evaporator. The residue was further purified by means of flash chromatography (silica gel 60; Merck; *n*-hexane + ethyl acetate = 5 + 1, v + v). The respective fractions containing the product were identified by their smell (drop on filter paper) and thin-layer chromatography. After removal of the mobile phase via a rotary evaporator (30 °C, 600 mbar), a semi-crystalline, slightly yellowish product with a strong smell of almond shell, cypress, and vanilla was obtained. The identity of the product was confirmed via EI (70 eV) and CI (115 eV) mass spectra (gas chromatograph: 5890 series II; Hewlett-Packard, Waldbronn, Germany; sector field mass spectrometer: MAT 95 S; Finnigan, Bremen, Germany) as well as by ^{1}H- and ^{13}C-NMR (Avance III 500 MHz ultrashield plus; Bruker, Rheinstetten, Germany).

Yield (raw): 79.5%
Yield (purified): 62.5%

EI spectrum *m/z* (%): 125 (100), 124 (44), 96 (64), 95 (58)
CI spectrum *m/z* (%): 126 (100), 125 (44), 112 (40), 111 (10)
^{1}H-NMR: (400 MHz, Methanol-d4) δ 5.56 (td, J = 2.6, 1.1 Hz, 1H); 5.03 (q, J = 1.4 Hz, 1H)
^{13}C-NMR: (101 MHz, Methanol-d4) δ 155.77; 137.45; 130.52; 129.36; 115.63; 110.57

Sample Pretreatment

In order to inactivate remaining yeast in the wheat beer samples, and therefore, prevent phenolic acids from further decarboxylation during sample workup, samples were pasteurised to 26 pasteurisation units (PU). Therefore, the wheat beer samples were incubated in a water bath at 70 °C. After reaching a temperature of 65 °C, the wheat beer samples were kept in the water bath for further 5 min to reach 26 PU. Subsequently, the pasteurised wheat beer samples were cooled to RT and stored at –18 °C prior to use. In addition, degassing of the wheat beer samples prior to work-up was not needed, as CO_2 could escape during fermentation via the air lock.

Sample Preparation for the Determination of Soluble and Free Phenolic Acids in Lab-Scale Fermented Wheat Beer Samples

Soluble Phenolic Acids (PAsol)

For the determination of PAsol (= sum of soluble ester-bound and free phenolic acids), wheat beer samples were treated in analogy to the sample preparation of wort samples described in section 3.2.2 [31].

Briefly, to access the soluble phenolic acid contents, ester-bound phenolic acids in the wheat beer samples had to be released via alkaline hydrolysis. Therefore, the stable isotopically labelled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were spiked to aliquots (0.5 mL) of the wheat beer samples. Next, a sodium hydroxide solution (0.5 mL, 4 mol/L) was added and the alkaline hydrolysis was performed for 2 h at RT with constant shaking (600 rpm; ThermoMixer; HLC HeizThermoMixer MHL23; Digital Biomedical Imaging Systems, Pforzheim, Germany). Subsequent to the incubation, the hydrolysates were neutralised by the addition of hydrochloric acid (0.5 mL, 4 mol/L), and the samples were thoroughly vortexed and membrane filtered (0.45 μm, Minisart RC, hydrophilic, 15 mm; Sartorius) prior to analysis by HPLC-MS/MS.

Free Phenolic Acids (PAfree)
PAfree were directly accessible from wheat beer samples. Samples were prepared in analogy to the work-up described in section 3.1.2 [30].
Briefly, aliquots (2 mL) of the wheat beer samples were spiked with the stable isotopically labelled internal standards (dissolved in H_2O + ACN (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments), followed by an equilibration for 15 min on a multitube vortexer (VWR, Darmstadt). Subsequently, the samples were membrane filtered (0.45 μm) and the phenolic acids were quantitated by means of HPLC-MS/MS.

High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS)

For the quantitation of cinnamic, *p*-coumaric, and ferulic acid, a HPLC-MS/MS system was used. For details, see section 3.2.2 [31].

Method Development for the Quantitation of Vinyl Aromatics in Wheat Beer

Quantitation of vinyl aromatics in wheat beer was performed by headspace-solid phase microextraction (HS-SPME) in combination with comprehensive gas chromatography-time-of-flight-mass spectrometry (GCxGC-ToF-MS). Concerning the HS-SPME method, the parameters fiber material (carboxen/polydimethylsiloxane (CAR/PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS)), extraction temperature (30, 35, 40, 45, and 50 °C), extraction time (5, 10, 20, 30, and 40 min), and salt content (1, 2, and 3 g) were optimised using a model solution consisting of deionised water, 5% of ethanol, and styrene, 4VP, and 2M4VP (0.1 mg/L each).
To check the applicability of the method, a validation was conducted including the determination of the limit of detection (LoD), the limit of quantitation (LoQ), and the recovery rate as well as a method comparison to an in-house method developed by Langos et al. [5], which was based

on high vacuum distillation using the solvent assisted flavour evaporation (SAFE, cf. Section 1.2.1) technique [9].

Limit of Detection (LoD) and Limit of Quantitation (LoQ)
LoDs and LoQs of styrene, 4VP, and 2M4VP were estimated from a pale wheat beer. The use of beer as matrix was shown to be important, as LoDs and LoQs from a model solution, only consisting of deionised water and 5% of ethanol, were found to be much higher. Very likely, this was due to the complex matrix present in wheat beer, acting as a kind of displacer occupying active sights of for example the used glassware, and thus preventing the adsorption of the analytes to these active sites. Therefore, the stable isotopically labelled internal standards were added and the spiked wheat beer (4VP and 2M4VP: 100, 50, 25, 10, 5, 2.5, 1, and 0 μg/L each; styrene: 10, 5, 2.5, 1, 0.5, 0.25, 0.1, and 0 μg/L) was diluted step by step with "unlabelled" wheat beer. The lowest concentration at which the peak of the stable isotopically labelled internal standard was still clearly observable, was specified as the LoQ, from which further the LoD was calculated based on the signal-to-noise ratio of 3:1 and 10:1 [23].

Recovery Rates
Recovery rates were determined using a pale wheat beer. Therefore, triplicates of this wheat beer were analysed once without the addition of the analytes and once spiked with the analytes styrene, 4VP, and 2M4VP, doubling the amount of the desired and undesired vinyl aromatics originally found in the pale wheat beer. After sample work-up, according to the newly developed method, vinyl aromatics were quantitated by means of HS-SPME-GCxGC-ToF-MS and recovery rates were calculated for each analyte.

Method Comparison
For method comparison, a wheat beer sample was analysed in triplicate once with the new SPME method and once with the in-house method (SAFE) developed by Langos et al. [5].

Sample Preparation for the Determination of the Desired and Undesired Vinyl Aromatics in Lab-Scale Fermented Wheat Beer Samples

For the determiantion of styrene, 4VP, and 2M4VP, wheat beer samples were prepared in analogy to the procedure described in section 3.1.2 [30].
Briefly, sodium chloride (2 g), tap water (4 mL), and wheat beer (1 mL) were mixed in a headspace vial (20 mL) equipped with a magnetic stir bar. Following, the stable isotopically labelled internal standards (dissolved in H_2O + ethanol (9 + 1, v + v); amounts depending on the concentrations of the analytes determined in preliminary experiments) were added, the headspace vial was sealed (silicon, PTFE septum screw caps), and the sample was stirred for 1 h on a magnetic stirrer at RT to adjust the sample headspace equilibrium.

Headspace-Solid Phase Microextraction-Comprehensive Gas Chromatography-Time-of-Flight-Mass Spectrometry (HS-SPME-GCxGC-ToF-MS)

For the quantitation of styrene, 4VP, and 2M4VP, a HS-SPME-GCxGC-ToF-MS system was used. For details, see section 3.1.2 [30].

Calibration lines were determined from mixtures of known amounts of the unlabelled analytes and the corresponding stable isotopically labelled internal standards in 7 different concentration ratios (10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10) with good linearities (R^2 >0.99) for all analytes in the applied ranges (Table 4.1).

Table 4.1: Selected quantifier ions (m/z), calibration line equations, and coefficients of determination (R^2) of vinyl aromatics and the corresponding stable isotopically labelled internal standards used for stable isotope dilution assays.

Analyte	Quantifier ion analyte [m/z]	Quantifier ion labelled standard [m/z]	Calibration line equation[a]	R^2
Styrene	104	112	0.716x + 0.151	0.997
4VP	120	125	1.258x – 0.053	0.999
2M4VP	150	153	0.568x – 0.013	0.999

[a] Determined via a 7-point-calibration using mixtures of known amounts of unlabelled analyte and corresponding stable isotopically labelled internal standard.

Statistical Analysis

Means and standard deviations were calculated using Microsoft Office 365 Excel (Microsoft Corporation, Seattle, WA, USA). Data visualisation and statistical analysis were performed applying the R statistical software (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria) [90] using the interface R Studio (version 1.1.423; RStudio, Boston, MA, USA) [91]. Scatter plots were created using the package ggplot2 (version 3.3.2) [92], regression lines were added using the function geom_smooth (method = lm), and correlation coefficients with p-values were calculated with the help of the function stat_cor (method = pearson) from the package ggpubr (version 0.3.0) [93].

4.2.3 Results and Discussion

Method Development for the Quantitation of Vinyl Aromatics in Wheat Beer

With the need for a high-throughput method for the quantitation of desired and undesired vinyl aromatics in wheat beer, a HS-SPME-GCxGC-ToF-MS method, including the technique of stable isotope dilution analysis, was developed. Thereby, HS-SPME was chosen for sample extraction, due to its capability for high-throughput analysis and GCxGC-ToF-MS was applied for quantitation to address the problem of overlapping peaks resulting from the very complex volatile fraction of wheat beer. Regarding the optimised HS-SPME parameters, the fiber material DVB/CAR/PDMS, an extraction temperature of 50 °C, an extraction time of 30 min,

and a salt content of 2 g were found to be the most efficient conditions for the extraction of styrene, 4VP, and 2M4VP from wheat beer.
Further, a method validation revealed that the respective LoDs and LoQs of styrene, 4VP, and 2M4VP were by far low enough to analyse the typical concentrations present in wheat beer. Finally, recovery rates of 73, 107, and 102% and recoveries within the method comparison of 103, 99, and 112% for styrene, 4VP, and 2M4VP, highlighted the suitability of the newly developed method for the quantitation of the target vinyl aromatics in wheat beer (Table 4.2).

Table 4.2: Validation results, including the limit of detection (LoD), limit of quantitation (LoQ), recovery rate, and a method comparison with the method of Langos et al. [5] for the determination of styrene, 4VP, and 2M4VP in wheat beer.

Analyte	LoD [μg/L]	LoQ [μg/L]	Recovery rate [%]	SPME vs. SAFE [%][a]
Styrene	1.50	5.0	73	103
4VP	0.75	2.5	107	99
2M4VP	0.03	0.1	102	112

[a] Recovery of the values of the SAFE method by the SPME method in percent.

Suitability of Lab-Scale Fermentation Experiments to Study Styrene Mitigation

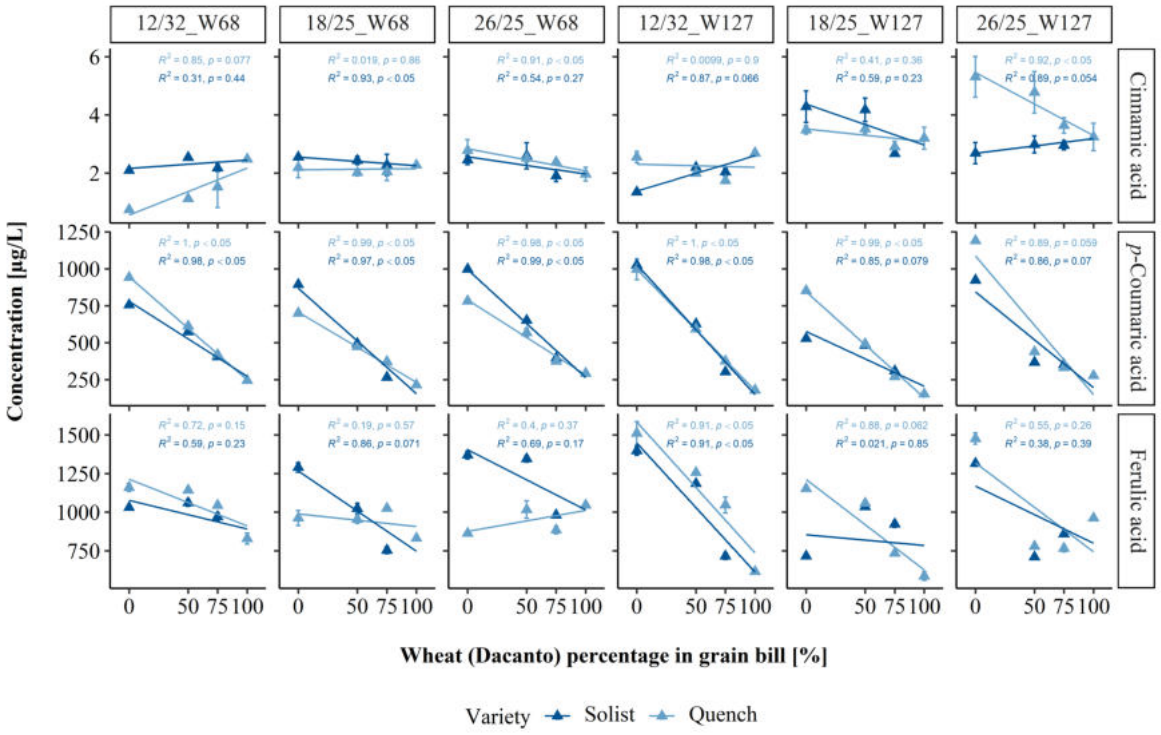
A comparison of the styrene contents of the lab-scale fermented wheat beers (0.7–5.3 μg/L, Figure 4.4a) with the contents found in the reference wheat beer (25.8±1.8 μg/L) as well as in the commercial wheat beers (9.8–31.6 μg/L) described in section 3.1.2 (see Table 2) revealed that styrene contents were approximately 5-10 times lower in the lab-scale fermented samples. This is most likely due to the fact that the lab-scale fermentations were carried out as unpressurised fermentations (open fermentation). This had the consequence that formed CO_2 could escape over the air lock, which may also has stripped off styrene. In fact, this was different to commercially available wheat beers, which are mostly fermented in pressurised tanks (closed fermentation). A study performed by Schwarz et al. [23], comparing an open and closed wheat beer fermentation, underpinned this assumption, showing only 25% of the styrene contents in wheat beer produced by open fermentation compared to closed fermentation.
Further, also the desired vinyl aromatics showed significantly lower contents in the lab-scale fermented wheat beer samples (4VP: 266–652 μg/L, 2M4VP: 710–1,350 μg/L (for reasons of comparability, data were presented from samples with 50 and 75% wheat malt in the grain bill), Figure 4.4a), with contents of approximately 50% of the concentrations found in commercial wheat beers (4VP: 560–1,350 μg/L, 2M4VP: 1,090–2,490 μg/L). In contrast, desired free phenolic acid contents were rather high in the lab-scale fermented samples (*p*-coumaric acid: 13–430 μg/L, ferulic acid: 114–1,650 μg/L (for reasons of comparability, data were presented from samples with 50 and 75% wheat malt in the grain bill), Figure 4.4b) compared to the commercial wheat beers (*p*-coumaric acid: 3–77 μg/L, ferulic acid: 11–353 μg/L). Free cinnamic acid contents were absent in both cases, which is related to the fact that free cinnamic acid

is already fully decarboxylated after 2 h of fermentation. In comparison, the desired vinyl aromatics need 3 to 6 days to reach their maximum levels [24]. Noticeable lower desired vinyl aromatic and higher free desired phenolic acid contents in case of the lab-scale fermentation experiments indicated that only a partial conversion of the free phenolic acids into the respective vinyl aromatics took place, rather compared to a full conversion of the free precursors as found in commercial wheat beers. In conclusion, the applied lab-scale fermentation conditions were proven to be improper when studying styrene mitigation in wheat beer brewed in industrial-scale.

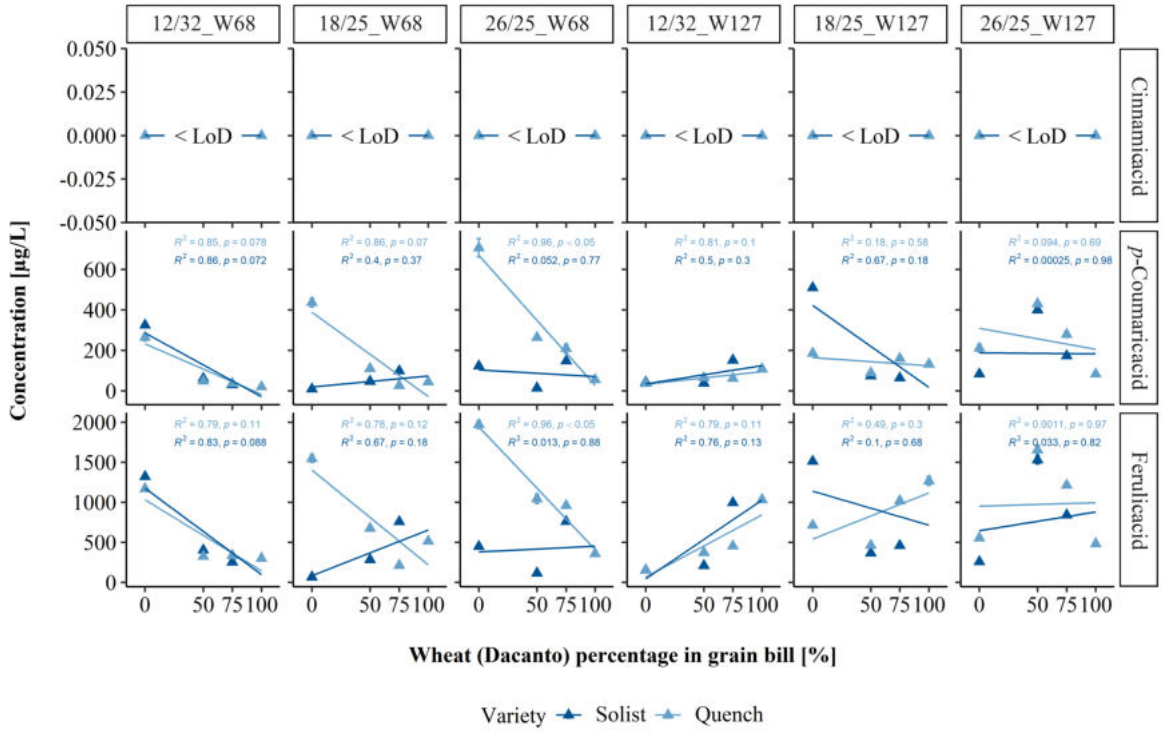
Findings from the Lab-Scale Fermentation Experiments

Although lab-scale fermentation was proven to be improper to study styrene mitigation, still some very interesting conclusions could be made from these experiments. Unexpectedly, soluble ester-bound cinnamic acid was still found in both the lab-scale fermented samples (21–128 μg/L, Figure 4.4c) and the reference wheat beer (30±1 μg/L). Of course, it is known that relevant soluble ester-bound amounts of the desired precursors are present after fermentation, however, it was assumed that in case of cinnamic acid the already very low soluble ester-bound contents are released by the yeast's own feruloyl esterase and are further decarboxylated. A possible explanation for the presence of ester-bound cinnamic acid might be given by the substrate specificity of the feruloyl esterase in yeast, which was already described in other microorganisms as very specific [94].

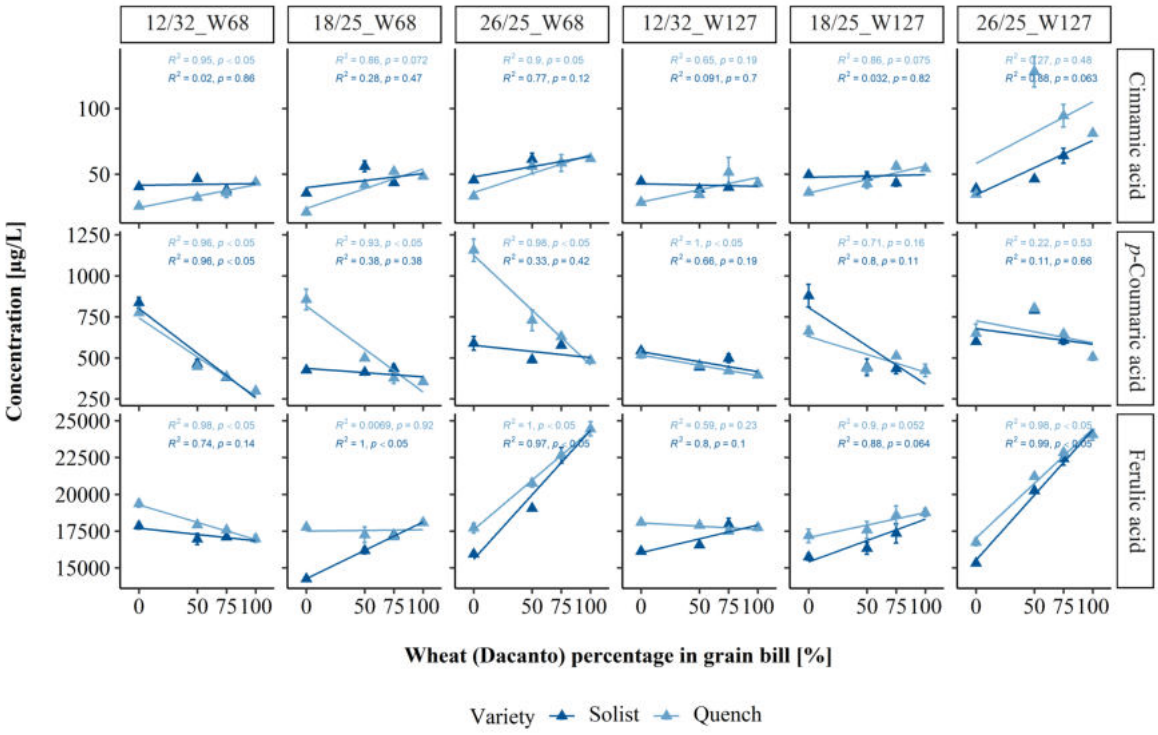
Regardless of styrene and 2M4VP, 4VP showed a clear trend in lab-scale fermented wheat beer samples with respect to the wheat malt content applied during mashing, independent of the malting parameters used for the preparation of the malts and the yeast strains added for fermentation (Figure 4.4a). With increasing wheat malt contents in the grain bill, a clear decrease of the 4VP contents was found. In fact, this clear dependency can be traced back to the huge differences in the contents of *p*-coumaric acid in barley and wheat, with up to 17-fold lower contents in wheat malts (cf. Section 3.2.2, Table 3) and 3-fold lower contents in wheat wort (cf. Section 3.5.2, Figure 1). These differences were way bigger than any effect caused by the malting parameters, the yeast strain, and the unsatisfying conditions during lab-scale fermentation. It is also expected that styrene and 2M4VP depend on the percentage of wheat malt in the grain bill and are linked to the amounts of the soluble and free precursors; however, conditions during fermentation have to be controlled more precisely to be able to study this coherence.



(a) Vinyl aromatic contents.



(b) Free phenolic acid contents.



(c) Soluble phenolic acid contents.

Figure 4.4: Phenolic acid and vinyl aromatic contents in lab-scale fermented wheat beer samples. Dependence of the vinyl aromatic contents (**4.4a**) as well as the contents of the free (**4.4b**) and soluble (**4.4c**) phenolic acids in lab-scale fermented wheat beer samples on the yeast strain (W68 and W127) used during fermentation and the percentage of wheat malt used during mashing (0:100, 25:75, 50:50, and 100:0).

Attempt to Unravel the Correlation Between Phenolic Acid Concentrations in Wort and the Final Vinyl Aromatic Concentrations in Wheat Beer

Aiming at understanding the correlation between the phenolic acid concentrations before and the vinyl aromatic concentrations after fermentation, soluble and free phenolic acid contents in wort, prepared from 50 and 75% wheat malt in the grain bill (closest to the wheat malt contents used in industry; for details, see section 3.5), were compared to the vinyl aromatic contents in the analysed reference wheat beer (Table 4.3). As the reference wheat beer had to be used for the calculations instead of the lab-scale fermented wheat beers, due to the above mentioned reasons, calculated values have to be seen as estimations.

In case of cinnamic acid, the free form was fully converted to styrene (0% PAfree in wort). However, at the same time, styrene contents in the reference wheat beer only accounted for 16% of the free cinnamic acid content in wort, whereupon approximately 80% of styrene formed during fermentation were lost. This finding was of interest, as it proved that the loss of styrene is not only an issue of open fermentation but also occurs during closed fermentation. If this is also due to evaporation is yet not answered. A different explanatory approach suspects

the plastic inside of the crown cork to be responsible for styrene adsorption, and therefore, for lowering its content also in wheat beers prepared by closed fermentation. Indeed, further research is needed to finally answer this question.
Similar to cinnamic acid, also free *p*-coumaric and ferulic acid were nearly completely converted into the corresponding vinyl aromatics, with only 2% of the free forms in wort left. However, now contrary to cinnamic acid, the soluble precursor and desired vinyl aromatic contents in the reference wheat beer accounted for roughly 100% of the soluble forms in wort, revealing that no losses of 4VP and 2M4VP occurred during fermentation. Deviations from 100% result from taking the values in the wort of section 3.5 for the calculations, instead of the corresponding not available wort of the reference wheat beer. This was highlighted by the mean percentages of 114% for *p*-coumaric acid and 88% for ferulic acid, when calculating the values for the lab-scale fermented wheat beers.

Table 4.3: Estimation of the percentage of soluble (PAsol) and free phenolic acids (PAfree) in wort[a] which were converted into vinyl aromatics[b] (VA) during fermentation and the percentage of precursors left.

	% PAfree from wort transferred into VA	% PAfree from wort left	% PAfree from wort which can be explained by PAfree and VA from wheat beer	% PAsol from wort transferred into VA	% PAsol from wort left	% PAsol from wort which can be explained by PAsol and VA from wheat beer
Cinnamic acid	16	0	16	12	10	23
p-Coumaric acid	125	2	128	86	74	159
Ferulic acid	42	2	43	6	64	70

[a] Wort data (50 and 75% wheat malt in grain bill) were taken from section 3.5. [b] Contents in commercially available wheat beers were represented by the data from the reference wheat beer.

Based on results obtained by Schwarz et al. [24], it is known that maximum styrene contents are reached already after 2 h of fermentation, whereas, depending on the fermentation temperature, the maximum contents of 4VP and 2M4VP are reached after 3 to 6 days. When reaching

their maximum, vinyl aromatics remain more or less constant at this level and form a plateau. Now taking into account that (i) free phenolic acids are decarboxylated to almost 100% during fermentation, (ii) soluble phenolic acid contents in wort match with the sum of the remaining soluble phenolic acid concentrations and the vinyl aromatic concentrations in wheat beer, and (iii) although vinyl aromatics reach a plateau while fermentation continues, soluble ester-bound phenolic acids are still present after fermentation, it was assumed that the final vinyl aromatic contents in wheat beer predominantly depend on the free precursor contents in wort, rather than on soluble ester-bound phenolic acid contents or the yeasts own feruloyl esterase activity. This hypothesis was further supported by a comparison of the soluble ester-bound phenolic acid contents in wort from section 3.5 and the contents found in the corresponding lab-scale fermented wheat beers, showing that the contents of the soluble ester-bound form stay rather constant during fermentation with recoveries of the contents before and after fermentation of 95, 125, and 93% for cinnamic, *p*-coumaric, and ferulic acid.

5 General Discussion

The enrollment of styrene into the compound class of carcinogens in 2002 [36] had a direct consequence for breweries. With styrene being a natural component of wheat beer, mitigation strategies had to be found to increase consumers safety. With former studies mainly focusing on the brewing steps after malting, this thesis specially focused on evaluating the ability of the malting process for the reduction of styrene via its precursor cinnamic acid.

5.1 Evaluation of the Status Quo in Commercial Wheat Beers

In a market survey, first, 18 commercially available pale wheat beers, two dark wheat beers, two reduced-alcohol wheat beers, two nonalcoholic wheat beers, and two bottom-fermented lager beers used as "negative" controls were analysed for their vinyl aromatic and free phenolic acid contents to get an overview of the typical concentration ranges of these analytes in wheat beer and how styrene contents correlate with the desired vinyl aromatic contents and the respective precursor concentrations. Further, the influence of dealcoholisation, roasting, and the yeast type on the analyte patterns was investigated. Therefore, respective high-throughput methods based on HPLC-MS/MS for the phenolic acids and HS-SPME-GCxGC-ToF-MS for the vinyl aromatics were developed and validated. Furthermore, as stable isotopically labelled 4VP was commercially not available, but needed for the stable isotope dilution assay, a new synthesis was successfully designed.

5.1.1 Vinyl Aromatic and Free Phenolic Acid Contents in Commercial Wheat Beers

To the best of our knowledge, this was the first time, that both the vinyl aromatic and the free phenolic acid contents were determined simultaneously in commercially available wheat beers. Within the analysed pale and dark wheat beers, styrene contents were found between 9.8 and 31.6 μg/L, 4VP contents between 434 and 1,350 μg/L, and 2M4VP contents between 620 and 2,490 μg/L, while the corresponding free precursor contents ranged from <0.8–3.4 μg/L for cinnamic acid, from 2.7–76.7 μg/L for *p*-coumaric acid, and from 11.3–353 μg/L for ferulic acid. Therefore, the typical analyte pattern of regular wheat beers was characterized by very high vinyl aromatic contents and rather low contents of the free precursors. Additionally, all wheat beers, eliciting the characteristic wheat beer aroma, revealed 2M4VP contents >620 μg/L. This

was in line with the observations of Wackerbauer et al. [8, 73], who assumed that wheat beers with a 2M4VP content <600 μg/L elicit an atypical to neutral wheat beer aroma.
A comparison to literature revealed that the found styrene contents in the present study were very similar to the contents found by two market surveys conducted in the years 1997 [20] and 2016 [28]. Styrene contents in these studies ranged between 25–31 μg/L and 15–33 μg/L, respectively. Concerning the question whether the implementation of the gained knowledge of former mitigation studies by breweries led to an improvement of the food safety of wheat beer, these data indicated that there was no reduction of styrene in most of the commercially available wheat beers over the past years, thus, highlighting the need for further studies on the complex topic of styrene mitigation.
Calculated Pearson correlation coefficients between and within the vinyl aromatics and the free phenolic acids revealed positive correlations within and negative correlations between the vinyl aromatics and precursors. Therefore, an increase of styrene in the analysed beer samples was always linked to an increase of the desired vinyl aromatics, and further, the increase of all three vinyl aromatics was related to a decrease of the free precursors. The causality of these correlations was linked to the presence of yeasts, whereby the negative correlations were the consequence of the ability of yeasts to convert free phenolic acids via decarboxylation into the corresponding vinyl aromatics [50, 51], and the positive correlations within the vinyl aromatics resulted from the lack of selectivity of the yeasts for the precursors [20]. If, however, the Pearson correlation coefficients were recalculated on the basis of a dataset including only the pale wheat beers, no significant correlations were found. This was very likely due to the fact, that wheat beer breweries in Bavaria usually use the same top-fermenting yeast strain for fermentation. Therefore, different ratios between styrene, 4VP, and 2M4VP among the analysed pale wheat beers were influenced by other factors, for example, the used barley and wheat variety [95, 96], the malting conditions [27], the barley to wheat malt ratio [21, 76], or the mashing conditions [22, 97]. All of them may affect the input of the precursors into the brewing process and at least also the fermentation management may affect the decarboxylation activity of yeasts [23, 24, 98].

5.1.2 Impact of Dealcoholisation, Roasting, and the Yeast Type on the Styrene Content in Wheat Beers

In principal, dealcoholisation of beer is achieved either by stopped fermentation when a certain alcohol level is reached or by removing the alcohol after a complete fermentation. Depending on the technique, different analyte patterns have to be expected. In case of a stopped fermentation, the reduced fermentation time was assumed to lead to higher free precursor concentrations and lower vinyl aromatic concentrations compared to a full fermentation. Only styrene should show comparable amounts to a full fermentation, as maximum styrene contents are known to be reached already after 2 h of fermentation [24], while 4VP and 2M4VP reach their maximum after 3–6 days [23]. If the alcohol is removed after a full fermentation, then free

precursor concentrations should be comparable to beers with regular alcohol content, whereas the vinyl aromatics, also including styrene, should show significant losses due to losses occurring during thermal evaporation. Based on these considerations, the impact of dealcoholisation on the analyte pattern was studied in comparison to a regular pale, a reduced-alcohol, and a nonalcoholic wheat beer originating from the same brewery. Thereby, the analyte pattern of the reduced-alcohol wheat beer matched the expected pattern (low precursor and vinyl aromatic contents in comparison to a full fermentation) when the technique of subsequent alcohol removal was used for dealcoholisation. However, a closer look at the beer parameters revealed that instead of a subsequent removal a lower original gravity was used by the breweries to reduce the alcohol content. With the lower original gravity, less precursors were introduced into wort, also leading to a reduction of styrene and the desired vinyl aromatics independent of a full fermentation. In fact, this strategy has a similar effect on the analyte pattern of a wheat beer as the technique of subsequent alcohol removal. The nonalcoholic wheat beer was prepared with a similar original gravity compared to the reduced-alcohol wheat beer. Now, with comparable styrene, lower 4VP and 2M4VP and higher free phenolic acid contents in the nonalcoholic wheat beer, clearly a stopped fermentation was used for its production.

The impact of roasting on the analyte pattern was studied comparing a regular pale and dark wheat beer originating from the same brewery. With styrene and desired vinyl aromatic concentrations in the dark wheat beer of approximately half the concentrations found in pale wheat beer, roasting was proved to have a strong impact on the vinyl aromatic concentrations. As both the dark and the pale wheat beer showed comparable low amounts of free precursors, indicating a full fermentation, and as similar original gravities were used for their production, lower styrene, 4VP, and 2M4VP concentrations must have been the consequence of a lower input of the precursors into the brewing process by the use of dark malts. In fact, a study of Samaras et al. [58] revealed that increasing kilning temperatures cause a massive loss of phenolic acids. With simultaneously increasing 2M4VP contents, the loss could be assigned to thermal decarboxylation occurring at higher kilning temperatures.

The ability of yeast to decarboxylate free phenolic acids into the corresponding vinyl aromatics was shown to be linked to the presence of the POF1 [51] and FDC1 [55] genes. Whereas bottom-fermenting yeasts lack without any exception the ability to decarboxylate phenolic acids, top-fermenting yeasts are an inhomogeneous group with either no, a reduced, or a high Pof-activity [20]. In order to study the impact of the yeast strain on the analyte pattern, a bottom-fermented lager beer and two top-fermented wheat beers were compared, all prepared from yeasts with different Pof-activities. In case of the bottom-fermented lager beer, very high free phenolic acid contents and negligible contents of the desired vinyl aromatics were found due to the missing decarboxylation ability. Styrene was even not detectable. Now, confirming the expectations, styrene and the desired vinyl aromatic contents increased starting from the bottom-fermented lager beer via the wheat beer with a reduced to the wheat beer with a high Pof-activity of the used yeast strains, while the free precursor contents decreased equally. In

fact, these results further pointed out the problem of the idea to use different yeast types for styrene mitigation, as this would inevitably be accompanied by a reduction of the desired vinyl aromatics, leading to wheat beers not eliciting the typical aroma expected by the consumers.

5.2 A General Understanding of the Impact of Malting and Mashing on the Free, Soluble Ester-Bound, and Insoluble Ester-Bound Cinnamic, *p*-Coumaric, and Ferulic Acid Contents

Specially focusing on the styrene mitigation ability of the malting process, next, this thesis worked towards a general understanding of the impact of malting and additionally also of mashing on the free, soluble ester-bound, and insoluble ester-bound cinnamic, *p*-coumaric, and ferulic acid contents and how they affected the ratio between the different forms and between the different phenolic acids. Therefore, malts and wort were prepared from four barley and three wheat varieties using typical conditions applied during wheat beer production. Additionally, stable isotope dilution assays were successfully developed for the determination of the total, soluble, and free precursor contents in grain, malt, and wort samples.

5.2.1 Changes in the Free, Soluble, and Total Cinnamic, *p*-Coumaric, and Ferulic Acid Contents upon Malting and Mashing

Since former studies on styrene mitigation were solely focused on the free form of the phenolic acids, the total and soluble cinnamic acid contents have so far not been reported in literature. Addressing this fact, the present study is, to the best of our knowledge, the first investigation on the maximum available cinnamic acid contents in grain, malt, and wort of different barley and wheat varieties. Total cinnamic acid contents in grain were found to range between 0.26 and 0.38 mg/kg dm and in malt between 0.86 and 1.27 mg/kg dm, while in wort, soluble contents ranged between 1.26 and 1.76 mg/kg dm. Thus, concentration differences of factors of up to 2,000 in malts and up to 100 in wort were found between the maximum contents of cinnamic acid and the total contents of *p*-coumaric and ferulic acid in these process intermediates. Based on these new information, it is not surprising that Langos et al. [28] monitored a massive release of the free desired precursors during mashing, while free cinnamic acid contents increased to a negligible degree, although, for example, cinnamic and *p*-coumaric acid showed comparable amounts in malt.

In case of cinnamic acid, all three forms were highly affected by malting and mashing, showing a constant increase from grain via malt to wort by factors between 1.3 and 12. Taking into account that the total cinnamic acid contents increased from grain to malt by factors of 2.9 till 3.9 and from malt to wort by factors of 1.3 till 2.2, malting had a stronger impact on the

maximum cinnamic acid contents than mashing, highlighting its important role in possible styrene mitigation. Regarding the amounts of cinnamic acid, independently of the phenolic acid form and the processing step, the barley varieties constantly showed slightly higher contents compared to the wheat varieties. However, as these differences were very low, a reduction of cinnamic acid, either by the use of different barley to wheat malt ratios or by the use of different varieties, was found to be very limited. For example, within the present dataset, the selection of a variety with a low content of the undesired precursor would have led to a reduction of cinnamic acid of <30%.

Different to cinnamic acid, malting only slightly affected the total *p*-coumaric acid content. While for the barley varieties the total contents did not change, an increase of 40–70% was found for the wheat varieties. This was different for the soluble and free forms of *p*-coumaric acid where malting and mashing had some decreasing and increasing effects. However, in total, these effects were negligible considering the huge concentration differences of total *p*-coumaric acid between unmalted barley and wheat. With these differences being in the range of roughly a factor of 10, soluble and free *p*-coumaric acid contents were directly related to the total contents, with always higher contents in case of the barley varieties.

Regarding ferulic acid, comparable total contents were found among the grain of barley and wheat varieties, which was similar to cinnamic acid, where also no significant concentration differences were present. Upon malting, a strong increase of the total ferulic acid content occurred, whereas at the same time, and now different to cinnamic acid, the contents of the soluble and free forms barely changed. With mashing, soluble ferulic acid contents decreased similarly in both grain types, with overall higher amounts in wheat wort. This was very interesting, as brewers agree to the fact that higher wheat malt contents in wheat beer brewing lead to a stronger wheat beer aroma. Langos et al. [29] already confirmed this empiric knowledge, determining significantly higher 2M4VP contents in wheat beers brewed from 100% wheat malt compared to beers brewed from 100% barley malt. Now, with the present data at hand, higher 2M4VP contents in wheat beers brewed from an increased percentage of wheat malt can be ascribed to overall higher ferulic acid contents, if wort was produced from higher wheat malt contents.

Based on the degradation of arabinoxylan during germination, found increasing total cinnamic, *p*-coumaric, and ferulic acid contents during malting were unexpected. However, this point of view neglects that cinnamic, *p*-coumaric, and ferulic acid are not solely responsible for the formation of arabinoxylan, but are also, as part of the phenylpropanoid metabolism, the major building blocks of secondary plant metabolites, such as lignin, flavanoids, and stilbenoids [59, 60]. Thus, an increase of the total amount of phenolic acids seems to be the consequence of the need of the seedling for these secondary plant metabolites to grow.

Furthermore, a comparison of the maximum phenolic acid contents between malt and wort of both grain types revealed transfer rates for cinnamic acid from 124–175%, but only from 2.6–8.0% for *p*-coumaric acid, and from 3.7–6.9% for ferulic acid. Transfer rates >100% in

case of cinnamic acid could not solely be explained by a complete transfer of the already free and soluble ester-bound forms in malt and by enzymatic activities during mashing allowing the release of remaining insoluble ester-bound cinnamic acid, but indicated that an additional biosynthesis of cinnamic acid during mashing must have taken place. This was different for *p*-coumaric and ferulic acid, where the rather small transfer rates of <8% were the consequence of the occurrence of these desired precursors predominantly in the insoluble ester-bound form in malt and of the activities of enzymes involved in the degradation process being too low to release higher amounts of insoluble precursors present in malt.

5.2.2 Effect of Malting and Mashing on the Ratio of the Different Phenolic Acid Forms

In grain, malt, and wort, very individual patterns were found concerning the ratios of the free, soluble ester-bound, and insoluble ester-bound forms of cinnamic, *p*-coumaric, and ferulic acid. Interestingly, these patterns proved to be very similar among the barley and wheat varieties. Starting with cinnamic acid, already in grain, the predominant form was the soluble form, whereby only a limited amount resulted from the free form. This changed upon malting where the soluble form increased in total; however, now with approximately half of it in the free form. The occurrence of cinnamic acid predominantly in a soluble form in grain and malt (Section 3.2.2 Figure 3) was unique among the target precursors and can be ascribed to the role of cinnamic acid in the phenylpropanoid metabolism, in which it is only an intermediate between L-phenylalanine and *p*-coumaric acid (Section 1.4.1 Figure 1.4) [59, 60]. In wort, finally, cinnamic acid occurred almost exclusively in the free form. Now, considering that in commercially available wheat beers no remaining free cinnamic acid contents could have been detected, that the total amount of cinnamic acid was transferred from malt into wort, and that mostly its free form was present in wort, a complete decarboxylation of cinnamic acid was assumed to take place during fermentation. According to this consideration, the formed amount of styrene should directly correlate with the concentration of its precursor. However, theoretical calculations revealed that only approximatley 20% of the formed styrene are later on present in wheat beer. Most likely, this is due to the loss of styrene via evaporation, which was already described by Schwarz et al. [23] causing lower styrene contents during open fermentation compared to closed fermentation. Furthermore, the very narrow distribution of the soluble cinnamic acid contents in wort of the barley and wheat varieties matched with the narrow concentration range of styrene in wheat beer found in the present market survey [30] and previously also by Langos et al. [28] and Daly et al. [20]. Thus, the styrene content in commercially available wheat beers is not expected to clearly exceed a maximum content of around 33 μg/L found in these studies.

Contrary to cinnamic acid, *p*-coumaric and ferulic acid predominantly occurred in the insoluble ester-bound form in grain (Section 3.2.2 Figure 3). Interestingly, malting had only minor effects

on the ratios, again with the insoluble ester-bound form as the most dominant form of the desired phenolic acids in malt, which further explained the low transfer rates of <8% in case of the desired precursors. After mashing, and thus finally in wort, a different situation was present for *p*-coumaric and ferulic acid. Whereas *p*-coumaric acid mostly occurred in the free form in wort, which was similar to cinnamic acid, ferulic acid was predominantly present in its soluble ester-bound form. This fact was related to the significantly higher amount of soluble ester-bound ferulic acid in wort that had to be released by the feruloyl esterase, as proven by a study of Schwarz et al. [25], showing that free ferulic acid constantly increased with a progressing mashing-in time, while cinnamic and *p*-coumaric acid reached a plateau very early in the mashing process.
Taking into account that in wort cinnamic and *p*-coumaric acid nearly exclusively existed in the free form, while ferulic acid was mostly present in its soluble ester-bound form and that cinnamic acid showed significantly lower contents compared to the desired phenolic acids, it can be assumed that maximum styrene concentrations are reached much faster during fermentation compared to 4VP and 2M4VP. Overall, the time to reach the maximum amount should be longest for 2M4VP, as not only higher amounts of free ferulic acid have to be converted by yeasts but also ferulic acid has to be released from soluble ester-bound forms. In fact, this was in accordance with the results of Schwarz et al. [23, 24], showing that maximum styrene contents are formed already after 2 h of wheat beer fermentation, whereas maximum contents of 2M4VP were reached after 3–6 days depending on the fermentation temperature.

5.2.3 Influence of Malting and Mashing on the Ratio of Cinnamic, *p*-Coumaric, and Ferulic Acid

Since during styrene mitigation sufficient amounts of the desired precursors have to be maintained to guarantee the formation of the characteristic wheat beer aroma, knowledge of the ratio between cinnamic, *p*-coumaric, and ferulic acid is of special interest. Investigations on this research question revealed significant differences between the barley and wheat varieties. In grain, the barley varieties revealed an average ratio of 0.1:37.2:62.7 for total cinnamic, *p*-coumaric, and ferulic acid, whereas the wheat varieties showed a ratio of 0.1:4.9:95.0. Thus, both grain types were characterised by similar low percentages of cinnamic acid of approximately 0.1%, whereas *p*-coumaric acid was found to contribute to a much higher degree to the overall phenolic acid content in case of the barley varieties compared to the wheat varieties. During malting of wheat, the phenolic acid contents increased to a similar degree, whereupon the ratio remained rather unaffected with 0.1:2.2:97.7. For the barley varieties, malting resulted in a ratio less dominated by *p*-coumaric acid of 0.1:15.2:84.7, as only the contents of cinnamic and ferulic acid increased, while the *p*-coumaric acid content did not change. During mashing, transfer rates >100% for cinnamic acid and <8% for *p*-coumaric and ferulic acid had the consequence that cinnamic acid now contributed to 1–2% of the phenolic acids present in wort of barley and wheat. At the

same time, *p*-coumaric and ferulic acid were similarly affected by mashing, whereupon the use of barley malt led to an introduction of cinnamic, *p*-coumaric, and ferulic acid into wort with a ratio of 1.7:13.7:84.6 and the use of wheat malt with a ratio of 1.2:2.0:96.8.

5.3 A Deeper Insight into the Biochemical Processes during Malting Directly or Indirectly Involved in the Release of Phenolic Acids

Aiming at a more detailed picture how malting affects the release of the precursors, a deeper insight into the biochemical processes, directly or indirectly involved in the release of the phenolic acids during malting, was of great interest. With this purpose, grain and malt samples of the same aforementioned four barley and three wheat varieties were analysed for their α- and β-amylase, β-glucanase, feruloyl esterase, and protease activities, as well as for their contents of β-glucan and total and water-extractable arabinoxylan. Assays needed for the determination of the enzyme activities and the nonstarch polysaccharide contents were successfully adapted or developed to answer this research question.

5.3.1 The Role of Cell Wall Degradation on the Release of Free or Soluble Ester-Bound Phenolic Acids

Two nonstarch polysaccharides, namely β-glucan and arabinoxylan, build up the cell walls in grain of barley and wheat. More precisely, the arabinoxylan forms a three-dimensional network into which the β-glucans are incorporated in form of bundles [99]. A complete degradation of these β-glucans during germination by the respective enzyme β-glucanase can, therefore, only occur if these bundles are released from this network and are thus accessible to the enzymes. In fact, this is only the case if the cell walls are degraded. Thus, the β-glucan contents were used as an indicator for the degree of the cell wall depletion and to evaluate whether a partial degradation of the cell walls plays a role in the release of phenolic acids. In grain, β-glucan contents were found to be much higher in the barley varieties compared to the wheat varieties. This confirmed a characteristic difference between these two grain types [100]. Upon malting, a strong degradation of the β-glucan content was observed, leading to similar low amounts in malts of the barley and wheat varieties. With this nonstarch polysaccharide structure being completely degraded, this was synonymous to a nearly complete depletion of the cell walls, suggesting that it is very unlikely that phenolic acids are trapped in intact cell walls. Regarding the activities of the corresponding enzyme β-glucanase, a reverse effect was found upon malting, starting with very low activities in the grain and ending up with very high activities in the malt. Further, in case of the barley varieties, high β-glucan contents in the grain were found to be linked to very high β-glucanase activities in the malts, whereas the lower β-glucan contents

in grain of the wheat varieties were found to correlate with also lower β-glucanase activities. This coherence was assumed to reflect the adaption of these two grain types to their β-glucan contents, guaranteeing, for example, the accessibility of starch as an energy source for the seedling by an efficient depletion of the cell walls.

5.3.2 The Role of the Solubilisation of Cell Wall Polysaccharies on the Release of Free or Soluble Ester-Bound Phenolic Acids

Linear chains of xylose, with arabinose irregularly attached, are the basic building block of arabinoxylan [62]. Via phenolic acids attached to the arabinose units, these linear chains are linked to a three-dimensional network [63, 64]. During malting, various enzymes degrade this network into fragments of different sizes [74]. According to their size, these fragments have a different solubility in water, and as they still contain phenolic acids, a transfer of phenolic acids into wort in a soluble but still ester-bound form can take place, which makes phenolic acids accessible for yeast later on in the brewing process. Therefore, the degradation degree of arabinoxylan was studied to answer the question to which extent the formation of water-extractable arabinoxylan correlates with the release of phenolic acids. Grain of barley and wheat revealed comparable amounts of total arabinoxylan, and upon malting, no significant change was found. This was very interesting, since the absence of significant differences in the total arabinoxylan content between grain and malt highlighted that possibly released xylose was not prone to a further metabolism. In an experiment determining the free xylose content, it could be shown that with $<0.5\%$ of the original arabinoxylan being present as free xylose, the degradation down to single sugar units is very unlikely to happen. Now, different to total arabionoxylan, water-extractable arabinoxylan contents were present at clearly higher amounts in grain of the wheat varieties compared to the barley varieties. Malting led to an increase of the water-extractable contents in both cases, however, with still higher amounts in wheat malts. Higher soluble ferulic acid contents in wheat wort compared to barley wort, prepared from the same malts, indicated that the degradation degree of the nonstarch polysaccharide arabinoxylan has an impact on the release of phenolic acids.

5.3.3 The Role of the Feruloyl Esterase Activity on the Release of Free Phenolic Acids

Besides the degradation of arabinoxylan during malting into fragments of different sizes, an additional enzyme, the so-called feruloyl esterase, directly cleaves off phenolic acids. Interested in whether the feruloyl esterase activities can be correlated with the free phenolic acid contents, its activity was determined in the grain and malts of the barley and wheat varieties. Thereby, feruloyl esterase activities were slightly higher in grain of the wheat varieties as compared to the barley varieties. During malting, the feruloyl esterase activity increased in both grain types, however, now with higher activities in malts of the barley varieties. With significantly higher

feruloyl esterase activities in barley malts and at the same time comparable total cinnamic and ferulic acid contents in malts of both grain types, barley malts were expected to show higher free phenolic acid contents. However, this was not the case, and thus, demonstrated that the feruloyl esterase activity is not exclusively responsible for the differences in the free phenolic acid contents and that additional factors, e.g., inhibitor proteins and total phenolic acid contents, contributed to these differences.

5.3.4 The Role of the Protease Activity on the Activities of Enzymes Directly or Indirectly Involved in the Release of Phenolic Acids

Proteases play a very important role in the growth of a seedling during germination. On the one hand, they degrade the protein matrix in which the starch granules are embedded, enabling the access of other enzymes to degrade starch as an energy source for the seedling, and on the other hand, they put free amino acids at the seedlings disposal. To what extent these proteases also affect the activities of the enzymes directly or indirectly involved in the release of phenolic acids, was further investigated. Calculated Pearson correlation coefficients revealed the absence of any correlation between the protease activity and the feruloyl esterase, α- and β-amylase, and β-glucanase activities, proving that the activities of these other enzymes were not affected by the protease activity. On the basis of their individual contribution to the growth of the seedling, it makes sense that they are not targeted by the proteases.

5.4 Structure Elucidation of Soluble Ester-Bound Phenolic Acids from Malt

Soluble ester-bound phenolic acids are thought to play a key role in the understanding how vinyl aromatic contents in wheat beer correlate with the respective phenolic acid contents in the process intermediates. In fact, this is related to its soluble nature, allowing phenolic acids, besides the free form, to end up from malt into wort during mashing, and thus, makes them accessible to yeast during fermentation. With very limited information on their structure in literature, a hot water extract of a wheat malt was analysed by means of UHPLC-ToF-MS for the purpose of structure elucidation. Therefore, the SWATH-MS technique, providing a data set with a fragmentation spectrum for every possible eluting substance was used. The fact that every possible fragment consists, apart from phenolic acid residues, solely of arabinose and xylose, and thus, must contain at least one mass loss, resulting from the cleavage of one sugar unit in its fragmentation spectra, and that oligosaccharides follow a distinct fragmentation pattern in mass spectrometry [84], four different fragments were found with m/z 589, 721, 853, and 1,117. Due to concentration reasons, all these fragments contained ferulic acid as the attached phenolic acid. Interestingly, a common building block was found among the four different fragments. The smallest of these fragments with m/z 589 was composed of two xylose

units, to which a ferulated arabinosyl unit was attached at the 3′-position at the reducing end. With increasing masses of the fragments, this structure was elongated at the reducing end by further xylose units. Based on the intensities of the respective fragments, a concentration ratio of 7:4:2:1 could be determined with increasing molecular weight (3, 4, 5, or 7 sugar molecules). A comparison to literature [84–86] confirmed the found fragment masses and postulated structures, which have been described as relatively short xylose chains with one ferulated arabinosyl unit.

5.5 Suitability of Selected Malting Parameters for Styrene Mitigation during Wheat Beer Brewing

On the basis of the new findings, selected malting parameters, such as the steeping temperature, the combination of germination temperature and aeration rate, and the temperatures applied during kiln-drying were evaluated in detail for their ability of styrene mitigation. For the malting experiments, two barley varieties and one wheat variety with industrial relevance were used, and the parameters total, soluble, and free phenolic acid contents, total and water-extractable arabinoxylan contents, and feruloyl esterase activity were determined.

5.5.1 Steeping Temperature

Investigations on the impact of the steeping temperature revealed decreasing cinnamic, *p*-coumaric, and ferulic acid contents in barley malts with increasing steeping temperatures (Section 3.4.2 Figure 1). This impact was further found to be strongest for cinnamic acid and dropped for *p*-coumaric and ferulic acid. Additionally, the steeping temperature seemed to have a slightly higher impact on the free form and to a lesser extent on the total and soluble forms. In the corresponding wort, again free and soluble cinnamic acid contents decreased with increasing steeping temperatures, whereas, now in contrast to the malts, *p*-coumaric and ferulic acid had their highest contents at 20 °C. Thus, in case of barley, higher steeping temperatures are clearly recommended to reduce styrene via its precursor cinnamic acid.
Wheat malts were differently affected by the steeping temperature, with the total, soluble, and free cinnamic, *p*-coumaric, and ferulic acid contents showing a clear formation at a temperature optimum of 20 °C. Upon mashing, again soluble and free *p*-coumaric and ferulic acid contents were found to be highest at 20 °C, whereas soluble and free cinnamic acid in the respective wort was lowest at this temperature. According to these results, in case of wheat, a steeping temperature of 20 °C seems to be favourable for styrene mitigation.
For the feruloyl esterase activity, no clear tendency was found with respect to the different steeping temperatures applied, and in consensus with former investigations, again no correlation was found between the feruloyl esterase activity and the free phenolic acid contents in the process intermediates (Section 3.4.2 Figure 2). In contrast, water-extractable arabinoxylan contents, clearly decreased with increasing steeping temperatures in barley malts, while they

showed their highest contents at 20 °C in the wheat malts (Section 3.4.2 Figure 2). Thus, also in consensus with former investigations, a correlation was found between the water-extractable arabinoxylan contents and the soluble and free phenolic acid contents in the respective malts. In summary, according to the present results, a proper selection of the steeping temperature would have the power to reduce the soluble cinnamic acid content in wort by up to 16%. Under these beneficial conditions, still sufficient amounts of the desired precursors were found, allowing the formation of the typical wheat beer aroma. Overall, the impact of the steeping temperature was rather small, which could be ascribed to the temperature dependency of the enzymes involved in the release of the phenolic acids, showing no huge differences in the applied temperature range.

5.5.2 Germination Temperature in Combination with the Aeration Rate

The technique of response surface modelling allowed the simultaneous study of the impact of the germination temperature and the aeration rate, both part of the germination step, on the release of phenolic acids during malting. In barley malts, water-extractable arabinoxylan contents decreased with increasing germination temperatures and decreasing aeration rates, with an overall higher impact of the germination temperature. Interestingly, in case of the wheat variety, the statistics revealed the water-extractable arabinoxylan content to be unaffected by these parameters. This was also the case for the feruloyl esterase activity in the barley varieties and the wheat variety.

Similar to the steeping temperature, trends in the water-soluble arabinoxyl content could be transferred to the dependency of the soluble and free phenolic acid contents on the germination temperature and aeration rate. Thus, the free phenolic acid contents in barley malts decreased with increasing germination temperatures, whereby the aeration rate played only a secondary role, and consequently, free phenolic acid contents were unaffected in malts of the wheat variety. Further, the impact on the total and soluble phenolic acid contents in the same malts was rather limited compared to the free phenolic acid contents.

Upon mashing, similar trends of the soluble and free phenolic acid contents were found in wort compared to the respective malts.

In conclusion, the present results recommended higher germination temperatures for the preparation of malts for wheat beer brewing when aiming at a reduction of cinnamic acid. Concerning the aeration rate, no clear recommendation can be made and apart from that, it seems to play only a secondary role regarding styrene mitigation. With the ability to reduce undesired cinnamic acid in wort by up to 50%, the germination temperature was proven to be a very powerful tool for styrene mitigation in the present study.

5.5.3 Kiln-Drying Temperatures

With the temperatures applied during kiln-drying being in the range where the most dominant differences are found in the temperature profiles of the enzymes capable for the degradation of arabinoxylan and of the feruloyl esterase, the withering temperature, part of kiln-drying, was expected to have the most pronounced impact on the precursor contents among the targeted malting parameters. In fact, the obtained results confirmed this expectation (Section 3.4.2 Figures 5 and 6). In malt, free cinnamic, *p*-coumaric, and ferulic acid contents were highest at a withering temperature of 60 ℃ and lowest at 30 ℃. In more detail, in case of the barley varieties, first, the free phenolic acid contents moderately increased from 30 ℃ to 50 ℃, followed by a striking increase from 50 ℃ to 60 ℃. Interestingly, this increase was more pronounced for cinnamic and *p*-coumaric acid than for ferulic acid. In case of the wheat variety, the increase of the free phenolic acid contents in malt was less distinct compared to the barley varieties; especially, the dominant increase from 50 ℃ to 60 ℃ was not found in wheat malts. Regarding the total and soluble phenolic acid contents in malts, in accordance with the results of the already discussed malting parameters, the impact of the withering temperature was less pronounced.

Water-extractable arabinoxylan contents in malts of the barley varieties were only slightly affected by the withering temperature, with the highest contents present at 50 ℃ and 60 ℃. For the wheat variety, statistics even revealed the absence of any effect. Overall, the withering temperature was able to change the water-extractable arabinoxylan content by not more than 24%. Thus, considering the differences in the phenolic acid contents of up to 84%, arabinoxylan degrading enzymes were only one of the factors explaining the overall changes.

Mashing of malts of both barley and wheat varieties led to wort with similar patterns of soluble and free cinnamic acid contents in dependency of the withering temperature compared to the respective malts. Therefore, the highest cinnamic acid contents in the barley varieties were again found at 60 ℃ and for the wheat variety, again comparable high contents were present at 50 ℃ and 60 ℃. With regard to the soluble and free *p*-coumaric and ferulic acid contents, the highest contents for both grain types were found in wort prepared at a withering temperature of 50 ℃, which was quite different to the patterns found in the corresponding malts. Very interestingly, also the feruloyl esterase activity in malt showed a temperature optimum of 50 ℃. Thus, with the feruloyl esterase being still active after kiln-drying, this seemed to be the first time that the feruloyl esterase activity outperformed other influencing parameters, showing a correlation to the phenolic acid contents in wort.

From the perspective of styrene mitigation, low withering temperatures are recommended during malt production according to the present results. Due to the biochemical pathways similarly targeting the phenolic acids, favourable conditions (cinnamic acid reduction) also reduce the *p*-coumaric acid content by up to 51% and the ferulic acid content by up to 31%. Fortunately, the amounts of the desired precursors were shown to be still high enough in these malts to form the desired wheat beer aroma. With the power to reduce undesired cinnamic acid in wort

by up to 72%, the withering temperature was proven to have the strongest impact among the analysed malting parameters, underlining its importance as a tool for styrene mitigation, in parallel with an aroma expected by the consumers.
As previously noted, dark wheat beers were proven to have significantly lower contents of the desired and undesired vinyl aromatics compared to pale wheat beers. At the same time, however, both have similar low amounts of the free precursors. According to the study of Samaras et al. [58], it was assumed that lower phenolic acid contents in dark malts, resulting from thermal decarboxylation due to the high kilning temperatures applied during kiln-drying of dark malts, might have caused the significantly lower vinyl aromatic contents. However, the analysis of malts kilned at 80 ℃ (pale) and 200 ℃ (dark) (Section 3.4.2 Figure 7) revealed that thermal decarboxylation is only one of the factors responsible for the lower styrene, 4VP, and 2M4VP concentrations in dark wheat beers. Interestingly, in these malts total, soluble, and free phenolic acid contents were only slightly affected by a kilning temperature of 200 ℃. Only in the corresponding wort, soluble and free phenolic acid contents were significantly lower when they were prepared from dark malts. Thus, the fact of slightly lower precursor contents in dark malts was not enough to explain the severe reduction in dark wort. In contrast, the lack of an additional release of *p*-coumaric and ferulic acid by the feruloyl esterase during mashing, an unusual lower transfer of already soluble phenol acids, and the absence of an additional biosynthesis, normally found for cinnamic acid, were found to cause the low phenolic acid contents in dark wort. Based on the missing feruloyl esterase activity in malts kilned at 200 ℃, causing the absent release of further phenolic acids during mashing, it was suggested that also other enzymes involved in the degradation of arabinoxylan and the biosynthesis of phenolic acids were affected by the high kilning temperatures applied during the preparation of dark malts.

5.6 Evaluation of the Role of the Barley to Wheat Malt Ratio Used for Wheat Beer Brewing in Styrene Mitigation

Already performed investigations highlighted the huge differences between the barley and wheat varieties with respect to their total, soluble, and free phenolic acid contents as well as their enzyme activity patterns. Since the barley and wheat varieties have always been studied separately up to now, this thesis addressed the question, whether the phenolic acid contents and enzyme activities of these two grain types may affect each other when mixing them for wheat beer brewing. Therefore, wort was prepared with different barley to wheat malt ratios (grain bill), and was further fermented with Pof-active top-fermenting yeasts, aiming at unraveling the contribution of the soluble ester-bound phenolic acids to the final vinyl aromatic contents in wheat beer.

5.6.1 Impact of the Barley to Wheat Malt Ratio during Mashing on the Free and Soluble Phenolic Acid Contents in Wort

During the analysis of wort prepared from different barley to wheat malt ratios, linear correlations were found between the soluble and free cinnamic, *p*-coumaric, and ferulic acid contents and the percentage of wheat malt in the grain bill (Section 3.5.2 Figures 1 and 2). In fact, this demonstrated that the phenolic acid contents were a direct function of their concentration in wort of 100% barley or wheat multiplied by the respective percentage of each grain type used for mashing. This further highlighted the absence of any synergistic effects caused, for example, by higher feruloyl esterase activities in barley combined with higher ferulic acid contents in wheat, when mixtures of barley and wheat malts were used for mashing.
Concerning barley to wheat malt ratios leading to a decrease of soluble and free cinnamic acid, the present results indicated that the decision whether to use higher or lower wheat malt contents for wheat beer brewing highly depends on the barley and wheat varieties used for mashing as well as on additional factors, such as the malting parameters. Both factors were also varied within these mashing experiments. In detail, depending on these factors, 100% barley and 100% wheat wort of the present investigation equally showed higher and lower cinnamic acid contents, which, based on the linear correlation, led to either increasing or decreasing cinnamic acid contents with an increasing percentage of wheat malt in the grain bill. This was different for soluble and free *p*-coumaric acid, where increasing wheat malt contents had a clear negative effect on the contents in wort, with the factors barley and wheat variety and malting conditions playing a negligible role. As already described before, this strongly pronounced dependency on the used grain type was a consequence of the characteristics of barley malts to show very high total *p*-coumaric acid contents, whereas the *p*-coumaric acid contents in wheat malts were found to be approximately 10 times lower. Finally, soluble ferulic acid showed an increase and free ferulic acid a decrease with increasing wheat malt contents. Responsible for these trends were the already described higher soluble and lower free ferulic acid contents in wheat wort and the contrary situation in barley wort, which were a consequence of different enzyme activities in these grain types. With comparable total ferulic acid contents in malts of barley and wheat, found higher arabinoxylan degrading enzyme activities in wheat malts led to higher soluble ferulic acid contents in wheat wort, and higher feruloyl esterase activities in barley malts led to higher free ferulic acid contents in barley wort. Further, the degree of the increase of especially the soluble form highly depended on the malting parameters, whereas the variety played only a minor role. This was due to the fact that increasing germination temperatures and decreasing aeration rates led to a stronger increase of the soluble ferulic acid contents in wort in case of the wheat variety compared to the barley varieties, causing a stronger difference in the contents between the two grain types. However, whether the soluble or free form is more relevant with respect to the formation of the vinyl aromatics in wheat beer brewing, is still unknown.

Furthermore, the impact of wort boiling was investigated, due to the higher temperatures applied. With the absence of relevant differences between unboiled wort and cast wort, wort boiling was proven to have no effect on the soluble and free phenolic acid contents in wort, and thus, temperatures applied within this process step are too low to cause relevant thermal decarboxylation. This observation was in line with a study performed by Samaras et al. [58], showing the absence of relevant amounts of 2M4VP in malts kiln-dried at temperatures <140 °C. Further, considering the high heat transfer capacity of water during wort boiling, a study by Langos et al. [28] also found no relevant differences in the contents of 4VP and 2M4VP between unboiled wort and cast wort.

5.6.2 Impact of the Barley to Wheat Malt Ratio during Mashing on the Free and Soluble Phenolic Acid Contents as well as the Vinyl Aromatic Contents in Lab-Scale Fermented Wheat Beer

Lab-scale fermentation in 50 mL PP-vials equipped with an air lock (open fermentation) was proven to be rather inappropriate to simulate and study industrial-scale wheat beer fermentation, mostly conducted in pressurised tanks (closed fermentation). This was the conclusion of a comparison of the target analyte patterns in the lab-scale fermented samples and a reference wheat beer, whereby 5 to 10 times lower styrene contents, significantly lower contents of the desired vinyl aromatics, and significantly higher contents of free phenolic acids were found for the laboratory-scale fermented wheat beer samples. The lower styrene contents could be ascribed to the used open fermentation, allowing styrene to be stripped off with CO_2 [23]. Additionally, the noticeable lower desired vinyl aromatic and higher free phenolic acid contents indicated that only a partial decarboxylation of the precursors took place in the lab-scale fermentation experiments.

Despite the fact that lab-scale fermentation was proven to be improper to study styrene mitigation, still some very interesting conclusions could be drawn from these experiments. First, still soluble ester-bound cinnamic acid contents were found in both the lab-scale fermented samples and the reference wheat beer. This was unexpected, as it was assumed that in case of cinnamic acid, the already very low soluble ester-bound contents were released by the yeast's own feruloyl esterase, and thus, were fully decarboxylated. A possible explanation for the present soluble ester-bound cinnamic acid contents might be given by the substrate specificity of the feruloyl esterase in yeast, which was already described in other microorganisms to be very specific [94]. Furthermore, 4VP, other than styrene and 2M4VP, showed a clear trend with respect to the wheat malt content in the lab-scale fermented wheat beer samples. Independent of the malting parameters used for the preparation of the malts and of the yeast strain added for the fermentation, 4VP contents decreased with increasing wheat malt contents in the grain bill. In fact, this clear dependency can be traced back to the already described huge concentration differences of *p*-coumaric acid between the barley and wheat varieties, which further outpaced

other effects such as the malting parameters, the yeast strain, and the inappropriate conditions during lab-scale fermentation. Very likely, also the contents of styrene and 2M4VP depend on the percentage of wheat malt used for mashing, and coupled to that, on the soluble and free precursor contents in wort; however, conditions during lab-scale fermentation have to be controlled more precisely in order to be able to prove this coherence. Finally, the very low styrene contents found in the lab-scale fermented wheat beers, with values 5-10 fold lower compared to the contents found in commercial wheat beers, are exactly what brewers are looking for.

Aiming at understanding the correlation between the phenolic acid concentrations before and the vinyl aromatic concentrations after fermentation, soluble and free phenolic acid concentrations in wort were compared to the vinyl aromatic concentrations in the analysed reference wheat beer. In case of cinnamic acid, the free form was fully converted to styrene. However, at the same time, styrene contents in the reference wheat beer did only account for 16% of the free cinnamic acid contents in wort, whereupon consequently 80% of styrene were somehow lost during fermentation. This fact was of interest, as it revealed that the loss of styrene is not only an issue of open fermentation [23], but also occurs in closed fermentation. Opposite to an open fermentation, evaporation is very unlikely to cause the loss of styrene in a closed fermentation, and thus, further explanations, as for example the adsorption of styrene to the plastic inside of the crown cork, have to be considered. Similar to cinnamic acid, also the free *p*-coumaric and ferulic acid contents were found to be nearly completely converted to the corresponding vinyl aromatics. However, now contrary to styrene, no losses of 4VP and 2M4VP occurred during fermentation, as the soluble precursors and desired vinyl aromatic contents in the reference wheat beer accounted for nearly 100% of the soluble contents present in wort. From a study of Schwarz et al. [24], it is known that maximum styrene contents are reached already after 2 h of fermentation, whereas the maximum contents of 4VP and 2M4VP are reached after 3 to 6 days. Further, after reaching their maximum, vinyl aromatics remain at this level and form a plateau. Now taking into account that (i) free phenolic acids are decarboxylated to almost 100% during fermentation, (ii) soluble phenolic acid contents in wort match with the sum of the remaining soluble phenolic acid concentrations and the vinyl aromatic concentrations in wheat beer, and (iii) although vinyl aromatics reach a plateau while fermentation continues, soluble ester-bound phenolic acids are still present after fermentation, it was assumed that the final vinyl aromatic contents in wheat beer predominantly depend on the free precursor contents in wort, rather than on soluble ester-bound phenolic acid contents or the yeasts own feruloyl esterase activity. This hypothesis was further supported by a comparison of the soluble ester-bound phenolic acid contents in wort and the contents found in the corresponding lab-scale fermented wheat beers, showing that the contents of the soluble ester-bound form stay rather constant during fermentation with recoveries of the contents before and after fermentation of 95, 125, and 93% for cinnamic, *p*-coumaric, and ferulic acid.

6 Conclusions

All in all, in the present study, malting has been demonstrated to be a very efficient tool for styrene mitigation in wheat beer brewing. First, mapping of the changes of the contents of total, soluble, and free cinnamic, *p*-coumaric, and ferulic acid upon malting and mashing allowed for a general understanding of the role of these processing steps in the release of phenolic acids. Further, both the total and soluble cinnamic acid contents were determined for the first time in grain, malts, and wort. Overall, the changes in the contents of the different phenolic acid forms, and consequently the changes in the ratios between the different forms and between the different phenolic acids, were the result of the contributions of the biosynthesis of the phenolic acids, their use within further catabolic pathways, the degradation of arabinoxylan, and the direct release of phenolic acids by feruloyl esterase to different extents. Thereby, compared to mashing, malting was proven to be of higher relevance in regard to styrene mitigation.
Secondly, the determination of the α- and β-amylase, β-glucanase, feruloyl esterase, and protease activities as well as of the contents of the nonstarch polysaccharides β-glucan and total and water-extractable arabinoxylan resulted in a deeper insight into the biochemical processes directly or indirectly involved in the release of phenolic acids during malting. For instance, it could be shown that due to the complete depletion of the cell walls during malting, it is very unlikely that phenolic acids are trapped inside intact cell walls. Only phenolic acids, either free or bound to water-soluble cell wall fragments, are later on accessible for yeasts. In this case, a clear dependency of the release of the phenolic acids on the degradation degree of the nonstarch polysaccharide arabinoxylan was observed. Interestingly, no correlation was found between the feruloyl esterase activity and the free phenolic acid contents in malts, demonstrating that the feruloyl esterase is not exclusively responsible for the free precursor contents and that additional factors, e.g., inhibitor proteins and the total phenolic acid contents, also play an important role. Calculated Pearson correlation coefficients revealed that enzymes directly or indirectly involved in the release of phenolic acids are not targeted by the grain's own proteases.
Thirdly, including four barley and three what varieties into these investigations, the characteristics of each grain type could be ascertained, showing great differences in the phenolic acid contents and enzyme activity patterns between barley and wheat. However, no synergistic effects were found by mixing barley and wheat malts for mashing. Instead of that, a linear correlation was found in dependency of the barley to wheat malt ratio. Thus, phenolic acid contents in wort for wheat beer brewing are a function of their concentrations in wort of 100% barley or wheat multiplied by the respective percentage of each grain type used for mashing.

Finally, with the main focus on the malting process and on the basis of the new findings, selected malting parameters were studied for their suitability for styrene mitigation. Thereby, conditions allowing for the reduction of undesired cinnamic acid in malts, and thus, also in wort, were identified. Aiming at styrene mitigation, the present results clearly recommended the use of high steeping temperatures, high germination temperatures combined with low aeration rates, and low withering temperatures when preparing barley malts and medium steeping temperatures, high germination temperatures combined with low aeration rates, and low withering temperatures when preparing wheat malts. Among the evaluated malting parameters, the withering temperature during kiln-drying was proven to have the strongest impact, with the capacity to reduce the soluble cinnamic acid content in wort by up to 72%, followed by the germination temperature combined with the aeration rate, showing a reduction capacity of up to 52%, with the aeration rate only playing a secondary role. The smallest impact, with a reduction capacity of 16% at most, was found for the steeping temperature, which could be ascribed to a temperature dependency of the enzymes involved in the release of phenolic acids, showing no great differences in the applied range. Indeed, conditions favourable for lower cinnamic acid contents also had a lowering effect on the contents of the desired precursors. However, overall still sufficient amounts of *p*-coumaric and ferulic acid were found in the respective wort, guaranteeing the formation of the desired aroma of wheat beer expected by the consumers.

In conclusion, this thesis aimed at a better understanding of the impact of malting of barley and wheat on the formation and release of the desired and undesired precursors of the vinyl aromatics during the production of wheat beer. Therefore, malting and mashing dependent changes in the total, soluble, and free cinnamic, *p*-coumaric, and ferulic acid contents were first studied in general, and additionally, enzymes directly or indirectly involved in the release of phenolic acids were examined in more detail. Since up to now, the barley and wheat varieties have always been studied separately, wort was subsequently prepared from different barley to wheat malt ratios to evaluate how phenolic acid contents and enzyme activities of these two grain types interact with each other when mixing them for wheat beer brewing. Further, the structures of soluble ester-bound phenolic acids in malt were elucidated and fermentation experiments with Pof-active top-fermenting yeasts were performed to unravel the contribution of the soluble ester-bound phenolic acids to the final vinyl aromatic contents in wheat beer. Finally, this project was successful in producing malts with a reduced content of undesired cinnamic acid by varying selected malting parameters. In the future, up-scaling of the malting experiments should prove whether the recommended conditions for styrene mitigation also lead to a reduction of styrene in wheat beer via its precursor cinnamic acid in an industrial-scale accompanied with the maintenance of the desired aroma expected by the consumers.

7 References

[1] Henrich, P. *Absatzverteilung von Bier in Deutschland nach Sorten 2019*; Statista: Hamburg, Germany, 2019; https://de.statista.com/statistik/daten/studie/307600/umfrage/absatzverteilung-von-bier-in-deutschland-nach-sorten/ (accessed 25.03.2020).

[2] Verband der Brauereienösterreichs. *Statistische Daten über die österreichische Brauwirtschaft*; 2018; https://bierland-oesterreich.at/unser-bier/wirtschaftsmotor/statistik/download/8-statistik/406-bierstat-2018.html (accessed 25.03.2020).

[3] Henrich, P. *Bierproduktion in Europa nach Ländern in den Jahren 2017 und 2018*; Statista: Hamburg, Germany, 2019; https://de.statista.com/statistik/daten/studie/175445/umfrage/bierproduktion-in-europa-nach-laendern/ (accessed 25.03.2020).

[4] Nijssen, L. M.; van Ingen-Visscher, C. A.; Donders, J. J. H. *VCF Volatile Compounds in Food 16.6.1*; Zeist: Reeuwijk, Netherlands, 2020; http://www.vcf-online.nl/ (accessed 26.3.2020).

[5] Langos, D.; Granvogl, M.; Schieberle, P. Characterization of the key aroma compounds in two Bavarian wheat beers by means of the sensomics approach. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 11303–11311.

[6] Grosch, W. Detection of potent odorants in foods by aroma extract dilution analysis. *Trends in Food Science & Technology* **1993**, *3*, 68–73.

[7] Schieberle, P. New developments in methods for analysis of volatile flavor compounds and their precursors. In *Characterization of Food: Emerging Methods*; Gaonkar, A. G., Ed.; Elsevier: Amsterdam, Netherlands, 1995; pp 403–431.

[8] Wackerbauer, K.; Krämer, P. Bavarian wheat beer - an alternative. Production and composition (in German). *Brauwelt* **1982**, *122*, 758–762.

[9] Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *European Food Research and Technology* **1999**, *209*, 237–241.

[10] Rychlik, M.; Asam, S. Stable isotope dilution assays for quantitation of organic trace

compounds in food analysis (in German). *Umweltwissenschaften und Schadstoff-Forschung* **2009**, *21*, 470–482.

[11] Rothe, M.; Thomas, B. Aroma of bread. Evaluation of chemical taste analysis with the aid of threshold value. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **1963**, *119*, 302–310.

[12] Tang, W.; Hemm, I.; Eisenbrand, G. Estimation of human exposure to styrene and ethylbenzene. *Journal of Toxicology* **2000**, *144*, 39–50.

[13] Murphy, P. G.; Macdonald, D. A.; Lickly, T. D. Styrene migration from general-purpose and high-impact polystyrene into food-simulating solvents. *Food and Chemical Toxicology* **1992**, *3*, 225–232.

[14] Choi, J. O.; Jitsunari, F.; Asakawa, F.; Lee, D. S. Migration of styrene monomer, dimers and trimers from polystyrene to food simulants. *Food Additives and Contaminants* **2005**, *7*, 693–699.

[15] Amirshaghaghi, Z.; Djomeh, Z. E.; Oromiehie, A. Studies of migration of styrene monomer from polystyrene packaging into the food simulant. *Iranian Journal of Chemical Engineering* **2011**, *4*, 43–49.

[16] Steele, D. H.; Thornburg, M. J.; Stanley, J. S.; Miller, R. R.; Brooke, R.; Cushman, J. R.; Cruzan, G. Determination of styrene in selected foods. *Journal of Agricultural and Food Chemistry* **1994**, *42*, 1661–1665.

[17] Fragnière, C.; Aebischer, J.-N.; Dudler, V.; Sager, F. A short study on the formation of styrene in cinnamon. *Mitteilungen aus Lebensmitteluntersuchung und Hygiene* **2003**, *94*, 609–620.

[18] Renner, R.; Bartels, H.; Tressl, R. Enrichment and quantitative determination of beer aroma substances by gas extraction (in German). *Monatsschrift für Brauerei* **1976**, *29*, 478–480.

[19] Tressl, R.; Friese, L.; Fendesack, F.; Koeppler, H. Gas chromatographic-mass spectrometric investigation of hop aroma constituents in beer. *Journal of Agricultural and Food Chemistry* **1978**, *26*, 1422–1426.

[20] Daly, B.; Collins, F.; Madigan, D.; Donelly, D.; Coakley, M.; Ross, P. An investigation into styrene in beer. *Proceedings of the Congress - European Brewery* **1997**, *26*, 623–630.

[21] Schwarz, K. J.; Methner, F.-J. Styrene concentrations during wheat beer production. *BrewingScience* **2011**, *64*, 156–158.

[22] Schwarz, K. J.; Boitz, L. I.; Methner, F.-J. Enzymatic formation of styrene during wheat

beer fermentation is dependent on pitching rate and cinnamic acid content. *Journal of the Institute of Brewing* **2012**, *118*, 280–284.

[23] Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Release of phenolic acids and amino acids during mashing dependent on temperature, pH, time and raw materials. *Journal of the American Society of Brewing Chemists* **2012**, *4*, 290–295.

[24] Schwarz, K. J.; Stübner, R.; Methner, F.-J. Formation of styrene dependent on fermentation management during wheat beer production. *Food Chemistry* **2012**, *134*, 2121–2125.

[25] Schwarz, K. J.; Boltz, L. I.; Methner, F.-J. Influence of mashing conditions on the release of precursors of phenolic wheat beer aroma. *Brauwelt* **2013**, *10*, 274–279.

[26] Langos, D.; Granvogl, M.; Gastl, M.; Schieberle, P. Influence of malt modifications on the concentrations of free phenolic acids in wheat and barley malts. *BrewingScience* **2015**, *68*, 93–101.

[27] Langos, D.; Granvogl, M.; Meitinger, M.; Schieberle, P. Development of stable isotope dilution assays for the quantitation of free phenolic acids in wheat and barley and malts produced thereof. *European Food Research and Technology* **2015**, *241*, 637–645.

[28] Langos, D.; Granvogl, M. Studies on the simultaneous formation of aroma-active and toxicologically relevant vinyl aromatics from free phenolic acids during wheat beer brewing. *Journal of Agricultural and Food Chemistry* **2016**, *64*, 2325–2332.

[29] Langos, D.; Gastl, M.; Granvogl, M. Reduction of toxicologically relevant styrene in wheat beer using specially produced wheat and barley malts. *European Food Research and Technology* **2017**, *193*, 558–568.

[30] Kalb, V.; Granvogl, M. Formation of desired smoky key odorants in wheat beer: a comparison with the undesired toxicologically relevant styrene. In *Sex, Smoke, and Spirits: The Role of Chemistry*; ACS Symposium Series 1321; Guthrie, B., Beauchamp, J. D., Buettner, A., Toth, S., Qian, M. C., Eds.; American Chemical Society: Washington, DC, 2019; pp 93–105.

[31] Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Studies on the impact of malting and mashing on the free, soluble ester-bound, and insolbule ester-bound forms of desired and undesired phenolic acids aiming at styrene mitigation during wheat beer brewing. *Journal of Agricultural and Food Chemistry* **2020**, *68*, 12421–12432.

[32] Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The role of endogeneous enzymes during malting of barley and wheat varieties in the mitigation of styrene in wheat beer. *Journal of Agricultural and Food Chemistry* **2020**, *68*, 13888–13896.

[33] Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. The malting parameters steeping, germination, withering, and kilning temperature and aeration rate as possibilities

for styrene mitigation in wheat beer. *European Food Research and Technology* **2021**, https://doi.org/10.1007/s00217–021–03852–5.

[34] Kalb, V.; Seewald, T.; Hofmann, T.; Granvogl, M. Investigations into the ability to reduce cinnamic acid as undesired precursor of toxicologically relevant styrene in wort by different barley to wheat ratios (grain bill) during mashing. *Journal of Agricultural and Food Chemistry* **2021**, *69*, 9443–9450.

[35] WHO *Guidelines for Drinking-Water Quality: Health Criteria and Other Supporting Information*; second Edition; World Health Organisation: Geneva, Switzerland, 1996.

[36] IARC *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: some Traditional Herbal Medicines, some Mycotoxins, Naphtalene and Styrene*; International Agency for Research on Cancer: Lyon, France, 2002.

[37] Bundesinstitut für Risikobewertung *Hinweise auf tumorauslösende Wirkung von Styrol beim Menschen*; Bundesinstitutes für Risikobewertung: Berlin, Germany, 2006; Vol. 25/2006.

[38] California Office of Environmental Health Hazard Assessment. *Safe drinking water and toxic enforcment act of 1986*; OEHHA: California, US, 2020; `https://oehha.ca.gov/proposition-65/` (accessed 02.10.2021).

[39] Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. *Toxicological Sciences* **1998**, *46*, 266–281.

[40] Cruzan, G.; Cushman, J. R.; Andrews, L. S.; Granville, G. C.; Johnson, K. A.; Bevan, C.; Hardy, C. J.; Coombs, D. W.; Mullins, P. A.; Brown, W. R. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *Journal of Applied Toxicology* **2001**, *21*, 185–198.

[41] Sumner, S. J.; Fennel, T. R. Review of the metabolic fate of styrene. *Critical Reviews in Toxicology* **1994**, *24*, 11–33.

[42] Ramsey, J. C.; Young, J. D. Pharmacokinetics of inhaled styrene in rats and humans. *Scandinavian Journal of Work, Environment & Health* **1978**, *4*, 84–91.

[43] Ramsey, J. C.; Young, J. D.; Karbowski, R. J.; Chenoweth, M. B.; McCarty, L. P.; Braun, W. H. Pharmacokinetics of inhaled styrene in human volunteers. *Toxicology and Applied Pharmacology* **1980**, *53*, 54–63.

[44] Vodicka, P.; Bastlova, T.; Vodickova, L.; Peterkova, K.; Lambert, B.; Hemminki, K. Biomarkers of styrene exposure in lamination workers: levels of O6-guanine DNA adducts,

DNA strand breaks and mutant frequencies in the hypoxanthine guanine phosphoribosyltransferase gene in T-lymphocytes. *Carcinogenisis* **1995**, *7*, 1473–1481.

[45] Artuso, M.; Angotzi, G.; Bonassi, S.; Bonatti, S.; de Ferrari, M.; Gargano, D.; Lastrucci, L.; Miligi, L.: Sbrana, C.; Abbondandolo, A. Cytogenetic biomonitoring of styrene-exposed plastic boat builders. *Archives of Environmental Contamination and Toxicology* **1995**, *29*, 270–274.

[46] Bastlova, T.; Vodicka, P.; Peterkova, K.; Hemminki, K.; Lambert, B. Styrene oxide-induced HPRT mutations, DNA adducts and DNA strand breaks in cultured human lymphocytes. *Carcinogenisis* **1995**, *16*, 2357–2362.

[47] Bastlova, T.; Podlutsky, A. Molecular analysis of styrene oxide-induced *hprt* mutation in human T-lymphocytes. *Mutagenesis* **1996**, *11*, 581–591.

[48] Carlson, G. P. Critical appraisal of the expression of cytochrome P450 enzymes in human lung and evaluation of the possibility that such expression provides evidence of potential styrene tumorigenicity in humans. *Toxicology* **2008**, *254*, 1–10.

[49] Gross, G. G. Biosynthesis and metabolism of phenolic acids and monolignols. In *Biotsynthesis and biodegradation of wood components*; Higuchi, T., Ed.; Academic Press: Orlando, FL, 1985; pp 229–271.

[50] Wackerbauer, K.; Kossa, T.; Tressl, R. Phenol formation by yeasts (in German). *Monatsschrift für Brauerei* **1978**, *31*, 52–55.

[51] Goodey, A. R.; Tubb, R. S. Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *Journal of General Microbiology* **1982**, *128*, 2615–2620.

[52] Meaden, P. G.; Taylor, N. R. Cloning of a yeast gene which causes phenolic off-flavours in beer. *Journal of the Institute of Brewing* **1991**, *97*, 353–357.

[53] Hwang, A. Restriction mapping of the POF1 gene. *Journal of the Institute of Brewing* **1992**, *98*, 467–470.

[54] Clausen, M.; Lamb, C. J.; Megnet, R.; Doerner, P. W. PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* **1994**, *142*, 107–112.

[55] Mukai, N.; Masaki, K.; Fujii, T.; Kawamukai, M.; Iefuji, H. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* **2010**, *109*, 564–569.

[56] Payne, K. A. P.; White, M. D.; Fisher, K.; Khara, B.; Bailey, S. S.; Parker, D.; Rattray, N. J. W.; Trivedi, D. K.; Goodacre, R.; Beveridge, R.; Barran, P.; Rigby, S. E. J.; Scrut-

ton, N. S.; Hay, S.; Leys, D. New cofactor supports α,β-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition. *Nature* **2015**, *522*, 497–501.

[57] Tressl, R.; Kossa, T.; Renner, R. Gas-chromatographic-mass spectrometric studies of volatile substances of hops, wort, and beer and their genesis. II. Formation of phenols and oxygen-containing heterocyclic compounds in beer. *Proceedings of the Congress - European Brewery* **1975**, *15*, 737–756.

[58] Samaras, T. S.; Camburn, P. A.; Chandra, S. X.; Gordon, M. H.; Ames, J. M. Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry* **2005**, *53*, 8068–8074.

[59] Hahlbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **1989**, *40*, 347–369.

[60] Ververidis, F.; Trantas, E.; Douglas, C.; Vollmer, G.; Kretzschmar, G.; Panopoulos, N. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. *Biotechnology Journal* **2007**, *2*, 1214–1234.

[61] Hermann, K. M.; Weaver, L. M. The shikimate pathway. *Annual Review of Plant Physiology and Plant Molecular Biology* **1999**, *50*, 473–503.

[62] Fincher, G. B. Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. *Plant Physiology* **2009**, *149*, 27–37.

[63] Gubler, F.; Ashford, A. E. Release of ferulic acid esters from barley aleurone. I. Time course of gibberellic-acid-induced release from isolated layers. *Australian Journal of Plant Physiology* **1985**, *12*, 297–305.

[64] Fincher, G. B. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annual Review of Plant Physiology and Plant Molecular Biology* **1989**, *40*, 305–346.

[65] Maillard, M.-N.; Berset, C. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. *Journal of Agricultural and Food Chemistry* **1995**, *43*, 1789–1793.

[66] Dvořáková, M.; Guido, L. F.; Dostálek, P.; Skulilová, Z.; Moreira, M. M.; Barros, A. A. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *Journal of the Institute of Brewing* **2008**, *114*, 27–33.

[67] Smith, M. M.; Hartley, R. D. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. *Carbohydrate Research* **1983**, *118*, 65–80.

[68] Adom, K. K.; Sorrells, M. E.; Liu, R. H. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Chemistry* **2005**, *53*, 2297–2306.

[69] Nordkvist, E.; Salomonsson, A.-C. Distribution of insoluble bound phenolic acids in barley grain. *Journal of the Science of Food and Agriculture* **1984**, *35*, 657–661.

[70] McNeil, M.; Albersheim, P.; Taiz, L.; Jones, R. L. The structure of plant cell walls VII. Barley aleurone cells. *Plant Physiology* **1975**, *55*, 64–68.

[71] Li, L.; Shewry, P. R.; Ward, J. L. Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 9732–9739.

[72] Andersson, A. A. M.; Lampi, A.-M.; Nyström, L.; Piironen, V.; Li, L.; Ward, J. L.; Gebruers, K. Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 9767–9776.

[73] Wackerbauer, K. Phenolic aromatic substances in beer. Phenolic carboxylic acid and phenols in the raw material and beer preparation (in German). *Brauwelt* **1982**, *122*, 618–620.

[74] Debyser, W.; Delvaux, F.; Delcour, J. A. Activity of arabinoxylan hydrolyzing enzymes during mashing with barley malt or barley malt and unmalted wheat. *Journal of Agricultural and Food Chemistry* **1998**, *46*, 4836–4841.

[75] Humberstone, F. J.; Briggs, D. E. Extraction and assay of ferulic acid esterase from malted barley. *Journal of the Institute of Brewing* **2000**, *106*, 21–29.

[76] Coghe, S.; Benoot, K.; Delvaux, F.; Vanderhaegen, B.; Delvaux, F. R. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces cerevisiae*. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 602–608.

[77] Jones, R. P.; Greenfield, P. F. Effect of carbon dioxide on yeast growth and fermentation. *Enzyme and Microbial Technology* **1982**, *4*, 210–223.

[78] Vanbeneden, N. Volatile phenols in beer: formation of 4-vinylguaiacol during wort fermentation and its fate during beer aging. *Procedings of the European Brewing Convention Congress* **2009**, Lecture 24.

[79] Wang, D.; Meng-meng, G.; Jin-hua, D. Determination and characterisation of feruloyl esterase in wheats and malts. *Food and Fermentation Industries* **2011**, *5*, 181–185.

[80] Back, W. *Ausgewählte Kapitel der Brautechnologie*; Fachverlag Hans Carl: Nuremberg, Germany, 2005.

[81] Pfenninger, H. *Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK)*; 3rd Edition; Brautechnische Analysenmethoden; Selbstverlag der MEBAK: Freising, Germany, 1997; Vol. 1.

[82] Krüger, E.; Anger, H.-M. *Kennzahlen zur Betriebskontrolle und Qualitätsbeschrei- bung in der Brauwirtschaft: Daten über Roh- und Hilfsstoffe, Halbfertig- und Fertigprodukte bei der Bierbereitung*; Behr: Hamburg, Germany, 1990.

[83] Gillet, L. C.; Navarro, P.; Tate, S.; Röst, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Molecular & Cellular Proteomics* **2012**, *11*, 1–17.

[84] Wang, J.; Yuan, X.; Sun, B.; Cao, Y.; Tian, Y.; Wang, C. On-line separation and structural characterisation of feruloylated oligosaccharides from wheat bran using HPLC-ESI-MS^n. *Food Chemistry* **2009**, *115*, 1529–1541.

[85] Malunga, L. N.; Beta, T. Isolation and identification of feruloylated arabinoxylan mono- and oligosaccharides from undigested and digested maize and wheat. *Heliyon* **2016**, *2*, 1–18.

[86] Malunga, L. N.; Eck, P.; Beta, T. Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono- and oligosaccharides from corn bran and wheat aleurone. *Journal of Nutrition and Metabolism* **2016**, *21*, 1–9.

[87] Farina, A.; Ferranti, C.; Marra, C.; Guiso, M.; Norcia, G. Synthesis of hydroxystilbenes and their derivatives via Heck reaction. *Natural Product Research* **2007**, *21*, 564–573.

[88] Albert, S.; Horbach, R.; Deising, H. B.; Siewert, B.; Csuk, R. Synthesis and antimicrobial activity of (*E*) stilbene derivatives. *Bioorganic & Medicinal Chemistry* **2011**, *19*, 5155–5166.

[89] Garcia-Barrantes, P. M.; Lindsley, C. W. Total synthesis of gombamide A. *Organic Letters* **2016**, *18*, 3810–3813.

[90] R Development Core Team. *A language and environment for statistical computing*; R Foundation for Statistical Computing: Vienna, Austria, 2019; `http://www.R-project.org` (accessed 14.4.2020).

[91] RStudio Team. *RStudio: integrated development for R*; RStudio: Boston, MA, 2019; `http://www.rstudio.com` (accessed 14.4.2020).

[92] Wickham, H.; Chang, W.; Henry, L.; Pedersen, T. L.; Takahashi, K.; Wilke, C.; Woo, K.; Yutani, H.; Dunnington, D. *R ggplot2: elegant graphics for data analysis*; 2020; `https://CRAN.R-project.org/package=ggplot2` (accessed 17.11.2020).

[93] Kassambra, A. *R ggpubr: based publication ready plots*; 2020; `https://CRAN.R-project.org/package=ggpubr` (accessed 17.11.2020).

[94] Williamson, G.; Faulds, C. B.; Kroon, P. A. Specificity of ferulic acid (feruloyl) esterases. *Biochemical Society Transactions* **1998**, *26*, 205–210.

[95] Vanbeneden, N.; Gils, F.; Delvaux, F.; Delvaux, F. R. Variability in the release of free and bound hydroxycinnamic acids from diverse malted barley (*Hordeum vulgare* L.) cultivars during wort production. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 11002–11010.

[96] Cui, Y.; Wang, A.; Zhang, Z.; Speers, R. A. Enhancing the levels of 4-vinylguaiacol and 4-vinylphenol in pilot-scale top-fermented wheat beers by response surface methodology. *Journal of the Institute of Brewing* **2015**, *121*, 129–136.

[97] Narziß, L.; Miedaner, H.; Nitzsche, F. The formation of 4-vinylguaiacol during the manufacture of Bavarian wheat beer (in German). *Monatsschrift für Brauerei* **1990**, *3*, 96–100.

[98] Kieninger, H.; Narziß, L.; Hecht, S. Changes of quality determining compounds in the preparation of Bavarian wheat beer (in German). *Monatsschrift für Brauwerei* **1984**, *1*, 9–18.

[99] Kunze, W. *Technology Brewing and Malting*; 5th Edition; Versuchs- und Lehranstalt für Brauerei: Berlin, Germany, 2016.

[100] Hübner, F.; Arendt, E. K. Germination of cereal grains as a way to improve the nutritional value: a review. *Critical Reviews in Food Science and Nutrition* **2013**, *53*, 853–861.

8 Acronyms

2M4VP	2-Methoxy-4-vinylphenol
4VP	4-Vinylphenol
AEDA	Aroma extract dilution analysis
BfR	Bundesinstitut für Risikobewertung
bp	Boiling point
C	Cinnamic acid
CAR	Carboxen
CI	Chemical ionisation
CoA	Coenzyme A
CYP	Cytochrome P450
Da	Dalton, unified atomic mass unit
DHS	Dynamic headspace
DIA	Data independent aquisition
dm	Dry mass
DNA	Deoxyribonucleic acid
DVB	Divinylbenzene
EI	Electron impact ionisation
ESI	Electrospray ionisation
F	Ferulic acid
FA	Formic acid
FD	Flavour dilution
FDC1	Ferulic acid decarboxylase
FEA	Feruloyl esterase
FID	Flame ionisation detection

GC	Gas chromatography
GC-O	Gas chromatography-olfactometry
GCxGC-ToF-MS	Comprehensive gas chromatography-time-of-flight-mass spectrometry
HPLC	High-performance liquid chromatography
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
HS-SPME	Headspace-solid phase microextraction
IARC	International Agency for Research on Cancer
LoD	Limit of detection
LoQ	Limit of quantitation
MS	Mass spectrometry
N_2	Nitrogen
NMR	Nuclear magnetic resonance spectroscopy
NSP	Nonstarch polysaccharide
NSRL	Non-significant risk level
OAV	Odour activity value
pC	*p*-Coumaric acid
PDMS	Polydimethylsiloxane
Pof	Phenolic off-flavour
POF1	Phenylacrylic acid decarboxylase
PP	Polypropylene
prFMN	Prenylated flavin mononucleotide
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PU	Pasteurisation Units
RI	Retention index
RT	Room temperature
S	Styrene
SAFE	Solvent assisted flavour evaporation

SIDA	Stable isotope dilution analysis
SPME	Solid phase microextraction
SWATH	Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra
TDI	Tolerable daily intake
TDU	Thermal desorption unit
THF	Tetrahydrofuran
ToF-MS	Time-of-flight-mass spectrometry
UHPLC	Ultra high-performance liquid chromatography
UHPLC-ToF-MS	Ultra high-performance liquid chromatography-time-of-flight-mass spectrometry
WHO	World Health Organisation