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HPLC-MS-based design of experiments approach on cocoa roasting

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ABSTRACT

Modern statistical methods, such as the design of experiments and response surface methodology, are widely used to describe changes in multiparameter processes during the processing of food in both science and technology contexts. However, these approaches are described to a lesser degree in the case of cocoa roasting than other foods and processes. Our study aimed to use the design of experiments to establish a model of cocoa roasting for relevant flavor-related constituents. We have used HPLC-MS techniques to link standard process parameters with chemical compounds changing in concentration during cocoa roasting. Influence of time, temperature, the addition of water, acid, and base, on relative concentrations of procyanidin monomers, dimers, and trimers, an Amadori compound, and a peptide, was shown. High-quality models for each compound were established and validated, displaying good prediction accuracy. Such an approach could be used to optimize processing conditions for cocoa roasting in order to influence the concentration of certain chemical compounds, and in turn, improving the flavor of chocolate products.

1. Introduction

Roasting of cocoa beans and thermal processing of food, in general, have a much longer history must be considered mostly an empirically driven process. It is common knowledge that applying different roasting conditions results in varying outcomes in terms of organoleptic properties, and these conditions were frequently optimized via simple methods such as "one-factor-at-a-time" (OFAT). Even though this method is still used to some degree, nowadays modern statistical techniques, such as the design of experiments (DoE) and response surface methodology (RSM) are becoming dominant in other fields of science and engineering. Both of these combined can provide enormous help with minimizing the number of experiments to be performed and maximizing the information output at the same time. Moreover, they are more and more used to describe processes both in food science and technology (Granato & de Araújo Calado, 2013). The benefits of these approaches were quickly recognized by, e.g. synthetic chemists, who no longer had to waste time optimizing their reaction yields by the OFAT approach (Leardi, 2009). The RSM proved to be very useful in analytical chemistry (Bezerra et al., 2008), even in the case of the development and optimization of mass spectrometric methods (Hecht et al., 2016). It could also be applied to optimize extraction conditions in metabolomic studies (Gullberg et al., 2004). Metabolomics can profit greatly from the design of experiments approach as they often involve work on big data sets with many variables involved (Jacyna et al., 2019), such as LC-MS-based metabolomics (Eliasson et al., 2012). The same reasons make the response surface methodology a valuable tool to improve industrial food processing (Yolmeh & Jafari, 2017). For example, it has been used during the optimization of roasting of various nuts to enhance their organoleptic properties (Özdemir & Devres, 2000; Kahyaoglu, 2008). On the other hand, the design of the experiments approach proved useful in the reduction of acrylamide formation, e.g. in bread, fries, and model systems (Bråthen & Knutsen, 2005; Mestdagh et al., 2008).

Similar optimization approaches could be applied to cocoa, which undergoes many complex chemical transformations on its way to becoming chocolate After cocoa roasting the chemical composition of a dried and fermented cocoa bean changes significantly. The chemical composition of dried fermented cocoa beans has been investigated in detail. 50–55% of the weight are lipids, composed from around 120 identified discrete molecules, in their majority triacylglycerides (Sirbu et al., 2018). Around 140 different polyphenolic secondary metabolites have been additionally described, being produced mainly as defense compounds in reaction to microbial fermentation (D'Souza et al., 2017). Carbohydrates are minor constituents with around 30 low molecular carbohydrates described and quantified (Megías-Pérez et al., 2018). During elevated temperatures at the point of fermentation, they already

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undergo significant chemical changes (Megias-Perez et al., 2020). For so-called aroma and flavor precursors, small oligopeptides must be considered the most relevant class of compounds. Around 800 such small oligopeptides, derived by proteolysis induced during fermentation have been recently described (D'Souza et al., 2018). The pH of a dried cocoa bean is slightly acidic due to lactic and acetic acid incorporation during fermentation. A top-down approach using ultra-high resolution mass spectrometry allows detection of around 10 000 signals in a dried fermented bean of which thus 20% have been attributed to defined structures of cocoa molecules (Kuhnert et al., 2020).

In this study, we focused on HPLC-MS assisted DoE approach to cocoa roasting. We explored how commonly used industrial roasting conditions influence the changes in the chemical composition of cocoa. So far, statistical approaches were not applied to cocoa roasting to the same degree as in the case of other foods and processes. However, among others, response surface methodology was used to explore the impact of roasting conditions on the sensory properties of chocolate (Rocha et al., 2017) and to optimize processing conditions towards maximum antioxidant activity and polyphenol content (Zzaman et al., 2014; Gültekin-Özgüven et al., 2016). The literature on other cocoa processing steps optimization and modeling seems to be richer, especially in the case of fermentation (Moreno-Zambrano et al., 2018; John et al., 2020). We believe that combining these approaches could not only help to optimize process parameters but as well shed some light on chemical transformation mechanisms during chocolate production.

2. Material and methods

2.1. Chemicals and reagents

Acetone, HPLC-grade acetonitrile, HPLC-grade isopropanol, HPLC-grade methanol, and dichloromethane were acquired from Carl Roth (Karlsruhe, Germany). Acetic acid, citric acid, formic acid, hesperetin, potassium carbonate, and sodium hydroxide were acquired from Sigma-Aldrich (München, Germany). Milli-Q water (18.2 MΩ•cm at 25 °C) was used during all the experimental work.

2.2. Nib preparation

A 25 kg bag of fermented and dried cocoa beans from the Ivory Coast was supplied by Barry Callebaut AG (Lebbeke-Wieze, Belgium). There was no additional contextual information provided for this sample.

A batch of approximately 200 g of cocoa beans was crushed using a small-scale cocoa bean crusher. A produced mixture of crushed beans (cocoa nibs) and shells was separated using a small-scale winnower. The winnowing process was repeated until the nibs were completely separated. The whole process was repeated until approximately 3.5 kg of cocoa nibs were obtained, which were kept in the fridge (4 °C) until further processing.

2.3. Experimental design and validation

We have recently demonstrated the success of the DoE approach to cocoa fermentation (John et al., 2020) and decided to apply a similar methodology to cocoa bean roasting. The whole process of design of experiments approach was done utilizing the MODDE Pro 11 software (MKS Umetrics AB, Sweden). The roasting model was designed to include temperature, time, humidity (water addition), and additives modifying pH (citric acid and potassium carbonate addition) as quantitative factors (see S-2 in the Supplementary Information for respective factors and their ranges). The additive factor combined both acid and base additives into one scale, with negatives values being acid concentration, positive being base concentration, and "0" being roasting without additives. Six constraints were defined for the model (see S-2 in the Supplementary Information). In principle the model applies to all cocoa constituents detectable by mass spectrometry (Milev et al., 2014), however, only five representative responses were chosen to be studied here, each being a concentration (expressed as relative areas) of a compound measured by HPLC-MS: epicatechin, procyanidin B-dimer, procyanidin B-trimer, an Amadori compound Fru-Phe, and a peptide VP. The first three compounds were reported as sums of concentrations of all their isomers. The model for each response employed multiple linear regression (MLR) and was orthogonally scaled. Initially, twenty-three model terms were included as a part of each model. The optimization (RSM) was directly selected as the objective of the design. The design space was explored using D-optimal design, and a model with the highest G-efficiency (parameter representing the variance of predicted values) (MODDE 11 User Guide, 2015) was selected from the ones generated by the software (see Fig. 1).

After performing sixty experiments designated by the software (as described in Section 2.4.), the models for each of the responses were refined by transforming the models and removing unnecessary model terms, in order to improve their fitting (measured by R2, Q2, validity, and reproducibility parameters, see Fig. 2F) (MODDE 11 User Guide, 2015). All the relevant descriptive statistics and model coefficients provided by the MODDE software are available in the Supplementary Information.

The refined models were validated using the optimizer function of the software. One objective per response was chosen (see Table 1), and the software generated the factor values to achieve a given objective, including the respective 95% confidence intervals. The objectives were chosen in such a manner to maximize desirable or minimize undesirable compounds affecting the cocoa flavor. The roasting experiments were performed in the same manner as the rest of the experiments (as described below, Section 2.4.).

2.4. Model roasting

The cocoa nibs were subjected to 60 model roasting experiments generated by the MODDE 11 Pro software according to the design. Approximately 50 g of cocoa nibs were placed on a metal tray with a large surface area to ensure uniform heat transfer and easier moisture evaporation during the roasting. Depending on the experiment, water, or water and an additive could be added to the tray and mixed thoroughly until homogenized. Additives (citric acid or potassium carbonate) were dissolved completely in water beforehand. The samples were roasted in a laboratory forced draft oven for a time and at a temperature specified by the software. Afterward, they were removed from the oven and left to cool down in a desiccator at room temperature. Roasted nibs were stored in a fridge at 4 °C until further use.

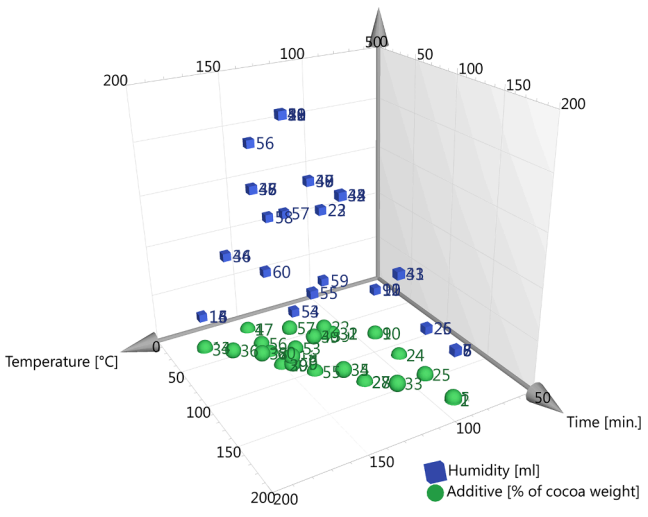

Fig. 1. Graphical representation of the design space. Each point on the graph represents a roasting experiment.

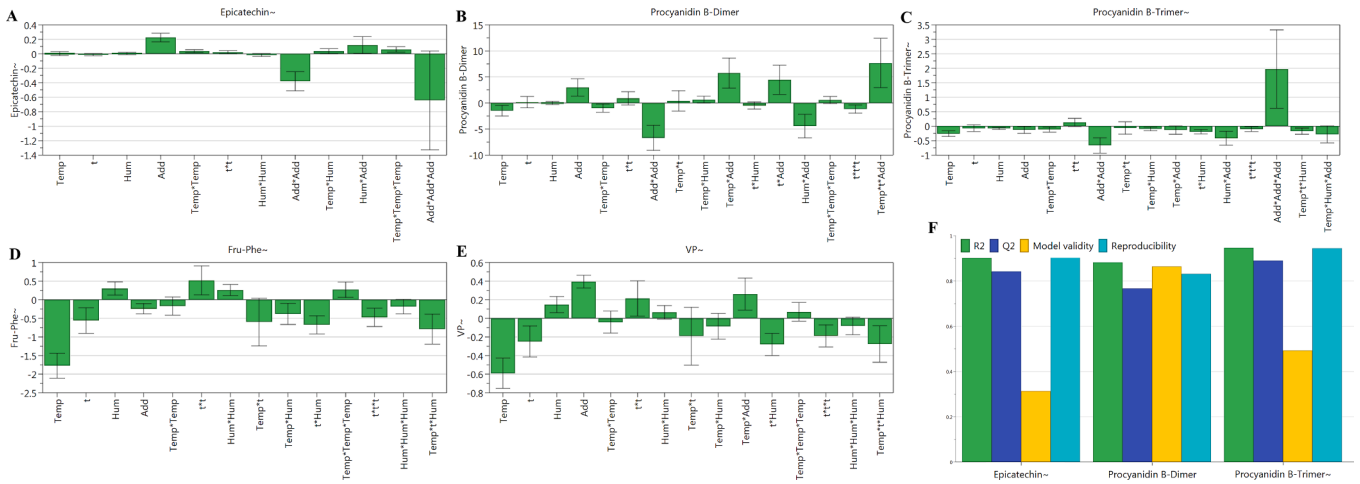

Fig. 2. The coefficients plot showing significant model coefficients (the error bars crossing the "0" indicate non-significant model terms) for: A) Epicatechin, B) Procyanidin B-Dimer, C) Procyanidin B-Trimer, D) Fru-Phe (fructosylphenylalanine, Amadori compound)., E) VP (dipeptide); F) Model statistics obtained for three of the responses (full graph in S-8 of the Supplementary Information).

Table 1
Validation experiments generated by the optimizer feature. Displayed predicted compound concentrations are relative MS peak areas.

Experiment number	Objective	Temperature [°C]	Time [min.]	Humidity (H_2O addition) [mL]	Additive [% of cocoa weight]	Epicatechin [Pred. rel. area]	Procyanidin B-Dimer [Pred. rel. area]	Procyanidin B-Trimer [Pred. rel. area]	Fru-Phe [Pred. rel. area]	VP [Pred. rel. area]
1	Maximize VP	114	56	26.06	0.98	15.83	5.69	3.07	2.22	1.52
2	Maximize B-Dimer	121	49	9.78	0.49	15.37	6.24	3.17	1.02	1.00
3	Minimize Epicatechin	146	87	47.15	−2.31	9.82	3.80	1.48	0.27	0.35
4	Minimize B-Trimer	146	86	47.43	2.29	14.96	3.22	1.01	0.16	0.91
5	Minimize Fru-Phe	173	41	1.34	0.03	15.87	3.24	1.41	0.03	0.24

2.5. Defatting and extraction

For each of the 60 samples, approximately 30 g of roasted cocoa nibs were ground to a fine powder at 10,000 rpm using a knife mill (Retsch Grindomix GM200, Germany). It was stored in a fridge at 4 °C before further use. About 6 g of the ground powder was defatted with dichloromethane in an automated Soxhlet extraction apparatus (Büchi B-811, Germany) for 18 h. The defatted powder was dried under the vacuum and stored at 4 °C before further experimental work.

The extraction of the defatted cocoa powder was carried out according to a previously established protocol (D'Souza et al., 2018). 50 mg of defatted cocoa powder was extracted with an acidified methanolic solution (MeOH:H2O:CH3COOH::70:28:2) by sonication in a Sonorex ultrasonication bath (Bandelin, Berlin, Germany) bath for 10 min and subsequent stirring for 30 min. The obtained extract was filtered through a PTFE syringe membrane filter (0.45 μm). Afterward, it was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and immediately used for HPLC-MS experiments.

2.6. HPLC-TOF-MS-MS measurements

HPLC conditions were adopted from a previously published method (D'Souza et al., 2018). Agilent 1260 HPLC system equipped with a ZORBAX Eclipse Plus C-18 column was used. Milli-Q water and acetonitrile with the addition of 0.05% of formic acid were used as Solvent A and Solvent B, respectively. The sample injection volume was set to 2 μL, constant flow rate to 0.4 mL/min, and the column oven temperature to 40 °C. The chromatographic gradient used for the analysis was the following: (t (min), %B): (0, 8); (1, 5); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (28, 95). The HPLC system was coupled to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (nebulizer pressure of 1.8 bars, the dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). The data were acquired in both positive and negative ion mode and calibrated in HPC mode. Reported NIST monoisotopic atomic masses of the elements (Coursey et al., 2015) were used to calculate monoisotopic molecular masses of detected compounds. 0.1 M sodium formate solution was used to calibrate the TOF analyzer before each sample measurement.

The methodology used for detection and analysis of all the cocoa constituents of interest was published previously for: procyanidin monomers, dimers, and trimers (D'Souza et al., 2017), Fru-Phe Amadori compound (Andruszkiewicz et al., 2020), and the VP peptide (D'Souza et al., 2018). The raw MS data was calibrated using Bruker Data Analysis 4.2 software and converted into .mzML format. All files were processed together as one batch using MZmine software. The obtained peak list with the area under the curve values was used further in the MODDE software analysis. The sum of all measured peaks for each sample was used to calculate the reported relative amounts of compounds. The relative concentration of each compound (epicatechin, procyanidin dimers and trimers, Fru-Phe, and VP) was calculated as a ratio of respective MS peak area to the sum of all peaks within the sample. In the case of epicatechin specifically, the relative amount is the sum of all the isomers with the same mass. The rest of the reported compounds are only single isomers. Processed data for each roasting experiment, expressed as relative MS peak area, is available in S-3 of the

Supplementary Information.

3. Results and discussion

Here we present a design of experiments approach applied to cocoa roasting, which in principle can be used both to optimize roasting conditions, as well as for mechanistic investigations of the roasting chemistry itself. Additionally, such studies could be valuable as a part of a larger predictive model, for example when coupled with similar approaches to cocoa fermentation (John et al., 2020).

The design of experiments process can be divided into three phases (design, analysis, and prediction) and will be described here as such.

3.1. Design phase

Typically, to model any process, in-depth knowledge of any parameters that can influence it is required. In order to do that, a separate screening design with a wide range of factors is built (MODDE 11 User Guide, 2015). However, as we mentioned, cocoa roasting is a long-established process, with its main parameters already known (Beckett, 2009). Therefore, we proceeded directly with an RSM (response surface methodology) optimization. The factors taken into the account in case of our roasting model were (see S-2 in the Supplementary Information): time, temperature, humidity (addition of water), and additives (addition of either citric acid or potassium carbonate).

These, except the acid addition, are the basic parameters commonly altered to produce different outcomes during the industrial roasting. Parameters used here include extreme under- and over-roasting boundary values in industrial roasting, which typically operates around 120 °C for 60–80 min. Additionally, there was a set of constraints imposed on the model to avoid any technical difficulties during the roasting (see S-2 in the Supplementary Information). The additive vs. humidity, to avoid the addition of either acid or base without addition of any water, and other humidity constraints promoted the water to be fully evaporated before the ending of the roasting (as in an industrial process). For this model, five responses were chosen to be studied by HPLC-MS: epicatechin (and its isomers), procyanidin B-dimer, procyanidin B-trimer, an Amadori compound Fru-Phe (fructosylphenylalanine), and a peptide VP. Each of them represents a class of compounds commonly known to participate in chemical transformations during the roasting of cocoa and being relevant to chocolate's taste. Epicatechins and procyanidins are polyphenolic compounds affecting astringency and bitterness (D'Souza et al., 2017). Amadori compounds, including Fru-Phe, are important flavor precursors, which can form in the course of fermentation and drying from the conjugation of sugars and amino acids or peptides (Andruszkiewicz et al., 2020). Peptides such as VP contribute to the generation of bitter 2,5-diketopiperazines during cocoa roasting (Andruszkiewicz et al., 2019; D'Souza et al., 2018; Frauendorfer & Schieberle, 2006; Frauendorfer & Schieberle, 2008; Granato & Ares, 2014; Hartmann & Schieberle, 2016; Stark & Hofmann, 2005). Among others, these compounds are potential quality markers for processing optimization of raw material.

The model for each response was based on multiple linear regression (MLR) and was orthogonally scaled. To maximize the predictive capabilities of the model, we added the maximum amount of model terms up to the order of three, including second-order interactions between the factors to the model terms (coefficients), twenty-three in total. This included first-, second-, and third-order terms for each of the factors (number of the order indicates the number of factors taken into account in the coefficient, e.g., time*time*time is a third-order term). After all the parameters of the design were established, a list of possible models was generated using the D-optimal method. From the available designs, the one with the highest G-efficiency (quality parameter) was selected. A graph of all the experiments generated within the chosen design, considering all the factors, constraints, and design parameters, is shown in Fig. 1. All the sixty roasting experiments were performed, and the results were investigated in the analysis phase.

3.2. Analysis phase

The HPLC-MS measurements provide the input for the responses, based on which the actual mathematical model can be constructed and refined. The summary of the fit graph (see Fig. 2**F**, values after refinement of the model) shows model quality for each response represented as four parameters: R2, Q2, model validity, and reproducibility (MODDE 11 User Guide, 2015). R2 describes the model fit, and the values of less than 0.5 suggest low significance (MODDE 11 User Guide, 2015). Q2 illustrates the predictive quality of the model, which as well should be above the 0.5 value (MODDE 11 User Guide, 2015). The model validity is an estimate of general model problems, like outliers or transformation problems, and each model should score at least 0.2 in model validity (MODDE 11 User Guide, 2015). The model reproducibility above 0.5 suggests reproducible results (MODDE 11 User Guide, 2015). Models for each of the responses show high values for every statistical quality parameter, which in turn suggests a high quality of each of the models. To achieve these results, logarithmic transformation was needed for some of the models. Additionally, to improve the quality of the models, some of the previously chosen model terms had to be removed. Fig. 2A-E shows all the model terms (coefficients) that are present in the refined model. Significant model terms (error bars of the respective term could not cross the zero on the coefficient plot) were kept, as well as the ones which changed the model quality in a major way upon deletion.

The established model enabled us to study both single-factor influences on the response, as well as the interactions between them. Fig. 3 shows the effects of additives on all the responses. The factor effects plots for temperature, time, and humidity can be seen in the Supplementary Information (S-6). The factor effects plot for time shows expected degradation trends for VP, Fru-Phe, procyanidin B-dimer, and B-trimer, with the most rapid degradations for the first two compounds, as they are reactive and participate in the Maillard reaction. While dimers and trimers degrade throughout roasting, epicatechin (and its isomers, e.g., catechin) is relatively stable. Unlike the rest of the compounds, epicatechin has a relative concentration uptrend when it comes to increasing temperature. This may be the result of the degradation of the procyanidin polymers. The humidity seems to have a minor effect on all the classes of compounds reported here. It is surprising, as higher water activity is reported to have an enhancing effect for various browning reactions (including Maillard reaction) (Eichnerl & Karel, 1987; Pereyra Gonzales et al., 2010). The most impactful of the factors appear to be additives, and it has the most diverse influence over all the responses. The Amadori compound (Fru-Phe, Fig. 3C) level is unaltered by either acid or base, whereas the epicatechin (Fig. 3A) and its trimer (Fig. 3E) seem to be affected only in most extreme conditions, as predicted by the model. The peptide (VP, Fig. 3D) degrades to a lesser extent basic conditions, and the procyanidin B-dimer (Fig. 3B) is stable only in neutral pH. This also is not consistent with the literature as compounds participating in the Maillard reaction should degrade faster with increasing pH (Cerrutti et al., 1985; Ajandouz & Puigserver, 1999; Martins & Van Boekel, 2003). However, the reports regarded model systems, avoiding any matrix effects specific to cocoa. Interestingly enough, the behavior of epicatechin and procyanidin B-dimer matches the one described in the literature, as catechins are stable in acidic pH and degrade readily in basic conditions, and the dimers are only stable in neutral pH (Zhu et al., 2002; Kırca et al., 2007).

The cumulative effects of each of the factors on the responses can be seen in the response surface plot shown in Fig. 4. As stated before, temperature and time factors have an expected influence on most of the responses, as anticipated in a thermal degradation process. On the other hand, the figure shows the most variability of each of the classes of compounds under different pH and humidity conditions. This is in line with the literature and industrial knowledge as these are the most common parameters used to control the course of browning reactions

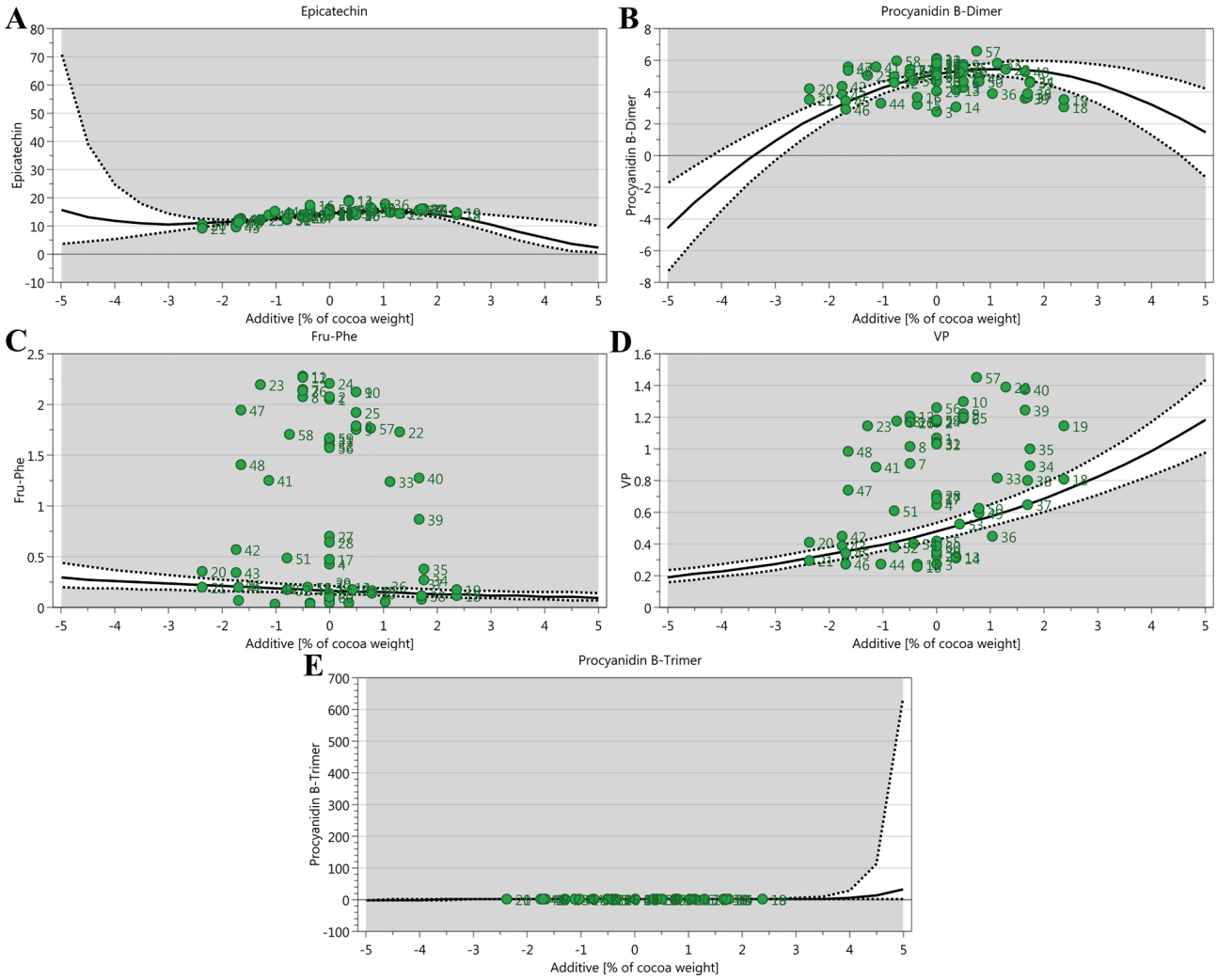

Fig. 3. Factor effects plot for "additive" as a response for: A) Epicatechin, B) Procyanidin B-Dimer, C) Fru-Phe (fructosylphenylalanine, Amadori compound)., D) VP (dipeptide), E) Procyanidin B-Trimer.

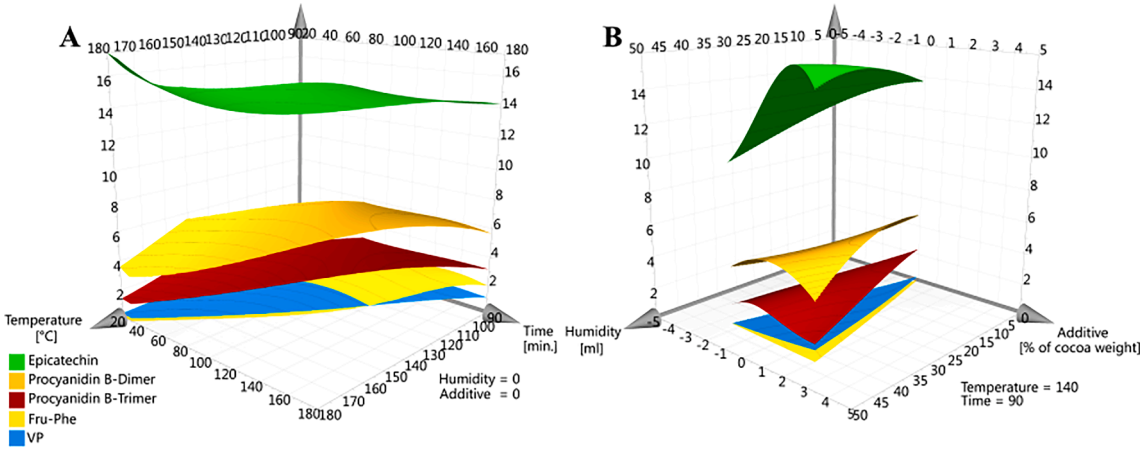

Fig. 4. Relationship between factors and responses represented by response surfaces. A) Temperature and time dependence is displayed (humidity and additives are constant), B) humidity and additive are shown (temperature and time kept constant).

(Martins & Van Boekel, 2003; Beckett, 2009; Pereyra Gonzales et al., 2010).

3.3. Prediction phase and model validation

The refined model was tested for prediction accuracy. Five experiments with distinctive objectives were planned (see Table 1). The

objectives were to maximize (VP and procyanidins) or minimize (epicatechin and Fru-Phe) concentrations (represented by MS relative areas) of five of the chosen compounds. We decided to optimize the roasting validation in such a way to yield the best flavor potential – maximize concentrations of precursors to unwanted compounds (degradation of VP peptide and procyanidins yield bitter 2,5-diketopiperazines and polyphenols), minimize the concentration of bitter epicatechin, and minimize the concentration of flavor precursor Fru-Phe (therefore maximization of degradation and conversion into desirable aroma compounds). The software then generated a list of experiments to be performed. Fig. 5 shows their results. Each optimization result for a single response was supplied with a set of predictions for the rest of the responses to explore the robustness of the model. The actual data for epicatechin, dimer, and trimer for experiment 1 is not shown because of poor MS calibration for this experiment. Aside from the comparison of predicted (with 95% confidence interval) and observed values, the figure shows the relative concentrations of respective compounds in the raw material represented by a line.

For most of the compounds and experiments, the established model estimated the values of relative concentrations very well. The one major outlier was the peptide (VP), which behaved quite differently than predicted during roasting. This could be because peptides are probably the most reactive compounds in the cocoa beans, and there are many competing reactions in which they can participate. The polyphenols have good predictability, and as well there is an option of pushing their balance toward either their conservation or degradation. There seems to be a balance between catechins and their oligomer counterparts, which would have to be explored in an expanded model, possibly including

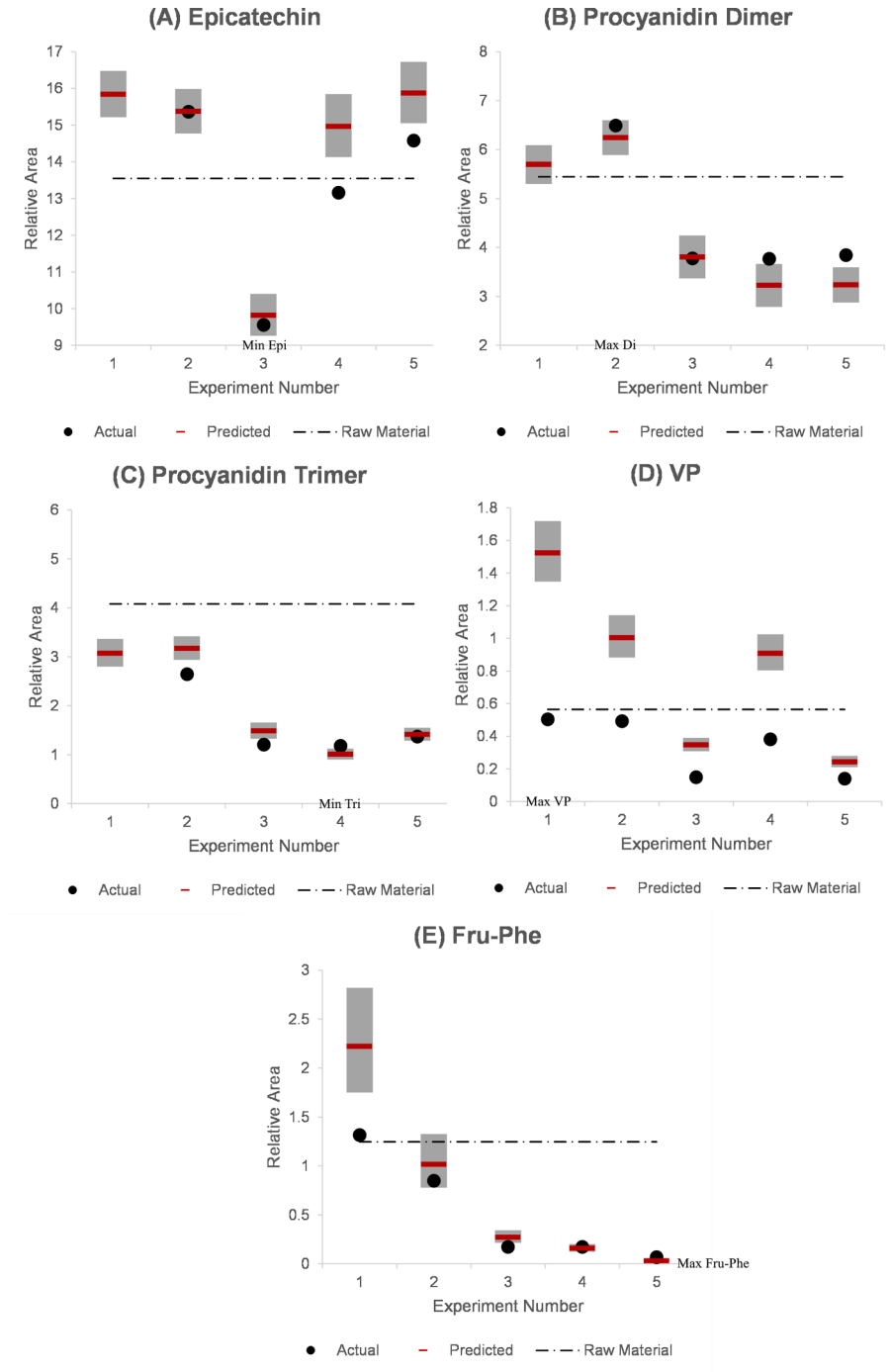

Fig. 5. Results of validation experiments for studied responses: A) Epicatechin; B) Procyanindin Dimer; C) Procyanidin Trimer; D) VP (dipeptide); E) Fru-Phe (fructosylphenylalanine, Amadori compound). The graph shows actual relative concentrations of all five responses against the predicted values (including 95% confidence intervals in gray) and the relative concentrations in the raw material. Each graph has annotation with an objective and a compound name by the respective experiment number (as referred to in Table 1).

higher oligomers. In the case of Fru-Phe, there is a possibility of decreasing its degradation with high accuracy. However, as its degradation is essential in the formation of aroma volatiles, this could most likely only decrease the quality of roasted cocoa. However, the validation of the model shows that most of the studied compounds' behavior is predicted very well. This approach, when expanded to different compound classes, could provide useful insights on chemical transformations and their mechanisms occurring during the roasting of cocoa.

4. Conclusions

In our study, we have applied common knowledge of cocoa processing to construct a design of experiments model of the roasting. We have illustrated it on an example of five well-known constituents of fermented and dried cocoa beans. Our results showed very good prediction and some unexpected behaviors of some of the compounds in comparison to established model systems knowledge. Nevertheless, we regard these initial results as very promising, especially for mechanistic studies of cocoa chemistry. Further expansions of this method could lead to optimization of cocoa processing, especially when coupled to similar, more systematized approaches of mathematical modeling of cocoa fermentation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors of this research paper receive research funding from Barry Callebaut. The terms of this arrangement have been reviewed and approved by the Jacobs University Bremen in accordance with its policy on objectivity in research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129694.

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