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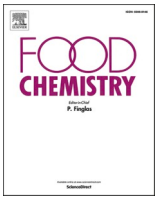

Characterizing the formation of process contaminants during coffee roasting by multivariate statistical analysis

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ABSTRACT

Coffee is a relevant source of dietary exposure for neoformed furan, alkyl furans and acrylamide. In this study, different statistical methods (hierarchical cluster analysis, correlation analysis, partial least squares regression analysis) were used for characterizing the formation of these process contaminants in green coffee beans roasted under the same standardized conditions. The results displayed a strong correlation between sucrose levels and furans in relation to the other sugars analyzed, while acrylamide formation was strongly related to the free asparagine. The data suggest that a sufficiently large amino acid pool in green coffee favors Maillard-induced acrylamide formation from asparagine, while reactions amongst the carbonyl-containing sugar fragmentation products leading to furan formation are suppressed. If the pool of free amino acids is small, it is depleted faster during roasting, thus favoring the formation of furans by caramelization, basically a sugar degradation process in which reactive carbonyl substances are generated and react together.

1. Introduction

Furan and methylfurans are volatile compounds occurring in a wide range of foods, especially those undergoing thermal processing (Crews & Castle, 2007; Fromberg et al., 2014; Scholz & Stadler, 2019). These compounds exhibit adverse health effects, and furan itself is classified by the IARC as a possible human carcinogen (group 2B) (IARC, 1995; Ravindranath et al., 1984). In 2017, the European Food Safety Authority published a scientific opinion on the health risks related to the presence of furan and methylfurans in food (EFSA, 2017) where the highest levels of these compounds were detected in coffee with a concentration up to 4500 μg/kg for furan and 3.5 to 4.4 times higher for 2-methylfuran. Despite the fact that furan and methylfurans are very volatile, substantial amounts are transferred to the coffee drink upon brewing (Rahn & Yeretzian, 2019). Thus, coffee was identified as a primary contributor to dietary exposure (EFSA, 2017).

Furan could be formed in food from a variety of precursors such as sugars, amino acids, unsaturated fatty acids, ascorbic acid and carotenoids (Becalski & Seaman, 2005; Owczarek-Fendor et al., 2011; Owczarek-Fendor et al., 2012; Perez Locas & Yaylayan, 2004; Shen et al., 2015). The levels of some of these compounds have already been determined in green coffee beans, where the level of sugars reaches up to 12.5% and are mainly composed of sucrose, and to a minor extent of fructose and glucose (Kim et al., 2021; Pavesi Arisseto et al., 2011). Whereas, the levels of free amino acids are almost 0.2–0.8% with aspartic and glutamic acid as the most abundant amino acids (Poisson

Abbreviations: AA, amino acid; DCM, dichloromethane; EI, Electronic Ionization; FMOC, 9-fluorenylmethyl chloroformate; GC, gas chromatography; HCA, hierarchical cluster analysis; HMDS, hexamethyldisilazane; HS-SPME, headspace-solid phase microextraction; OPA, *ortho*-phthalaldehyde; PC, process contaminants; PLS, partial least squares; PTV, Programmable Temperature Vaporization; S, sugars; SPE, solid phase extraction; TCA, Trichloroacetic acid.

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et al., 2017).

Several mechanisms for furan formation have been already suggested such as thermal degradation of carbohydrates or amino acids, Maillard reaction between reducing sugars and amino acids, and the oxidation of polyunsaturated fatty acids or ascorbic acid (Batool et al., 2021; Crews & Castle, 2007). However, these mechanisms have been suggested in simplified model systems in which the complexity of a real food matrix cannot be fully mimicked (EFSA, 2017).

Acrylamide is another relevant probably carcinogenic process contaminant (Group 2A) (IARC, 1994) which is formed in a variety of heated foods, including coffee. The free amino acid asparagine has been identified as the main precursor, which during the Maillard reaction is converted to the corresponding Amadori product which upon decarboxylation and deamination is converted to acrylamide (Mogol & Gökmen, 2016). As it is water soluble, it is transferred to the coffee drink upon brewing (Bagdonaite et al., 2008).

The amount of furan formed during the roasting process is related to the roasting conditions under which the reaction takes place (Cha & Lee, 2020; Mogol & Gökmen, 2013). However, no studies are reported in which a substantial amount of different coffee samples with a variable content of precursors for the considered process contaminants have been subjected to a standardized roasting process in order to investigate the link between the concentration of the precursors in the green beans and the concentration of the process contaminants in the roasted beans. Therefore, the aim of this study was to establish the potential link(s) between chemical composition (amino acids and sugars) and process contaminants (furan compounds, thiophene compounds, acrylamide) in coffee samples with different geographical origin, variety (Robusta vs. Arabica) and caffeine content. Thiophenic compounds, namely thiophene (C_4H_4S) and 2-methylthiophene (C_5H_6S) were included in the present study because of their structural similarity to the furans under consideration. Furthermore, potential differences due to geographical and compositional parameters were assessed. To achieve the aforementioned aims, different multivariate statistical methods were applied. Firstly, agglomerative hierarchical cluster analysis (HCA) was applied for evaluating the similarity between individual coffee samples or the studied variables. Secondly, Spearman correlation analysis was applied for assessing the correlations between the studied variables. Finally, partial least squares regression (PLS) was applied for identifying chemical constituents that could be used for predicting the levels of selected process contaminants.

2. Materials and methods

2.1. Materials and chemicals

2.1.1. Green coffee samples

64 batches of green coffee of different geographical origin, type (12 Robusta, 51 Arabica, 1 blend) and caffeine content (55 caffeinated, 9 decaffeinated) were used in this work (Table 1). All these samples were provided by Belgian coffee suppliers or traders and stored at room temperature until analysis. Two portions were taken from each batch for further investigations. A portion of 25 g was used for the analysis of amino acids and sugars in green coffee. This portion was ground to obtain fine homogeneous powder and stored in a closed container at room temperature prior to analysis. The other 500 g portion was roasted as described in Section 2.2.

2.1.2. Standards and chemicals

Analytical standards for twenty-two amino acids (list in Table 2, norvaline and sarcosine), six sugars (D-fructose, D-glucose, sucrose, D-maltose, β-lactose and phenyl-*b*-D-glucopyranoside), furan and five alkylfurans (2-methylfuran, 3-methylfuran, 2-ethylfuran, 2,5-dimethylfuran and 2,3-dimethylfuran) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiophene and 2-methylthiophene were bought from VWR (Radnor, PA, USA) and acrylamide from Chiron (Trondheim, Norway). For labelled standards analogous, furan and alkyls (furan-d_4, 2-methylfuran-d_6, 3-methylfuran-d_3 and 2,5-dimethylfuran-d_3) were bought at Toronto Research Chemicals (Toronto, ON, Canada), while acrylamide (acrylamide-d_3) comes from Chiron.

Methanol HPLC grade, boric acid, trifluoroacetic acid (TFA), hexamethyldisilazane (HMDS) and Carrez Solution II (30% ZnSO4) were

Table 1
The coffee samples: sample ID (according to Beyers), geographical origin, type and caffeine content. Notation indicates the type (A = Arabica, R = Robusta) and caffeine content (Y = yes, N = no).

Sample (n)	Origin	Type	Caffeine	Notation
1	Peru	Arabica	Yes	A-Y
2	Honduras	Arabica	Yes	A-Y
3	Mexico	Arabica	Yes	A-Y
4	Guatemala	Arabica	Yes	A-Y
5	Brazil	Arabica	Yes	A-Y
6	Mexico	Arabica	Yes	A-Y
7	Honduras	Arabica	Yes	A-Y
8	Nicaragua	Arabica	Yes	A-Y
9	Ethiopia	Arabica	Yes	A-Y
10	Kenya	Arabica	Yes	A-Y
11	Guatemala	Arabica	Yes	A-Y
12	Rwanda	Arabica	Yes	A-Y
13	Nicaragua	Arabica	Yes	A-Y
14	Honduras	Arabica	Yes	A-Y
15	Santos	Arabica	Yes	A-Y
16	Santos	Arabica	Yes	A-Y
17	Colombia	Arabica	Yes	A-Y
18	Congo	Arabica	Yes	A-Y
19	Aqua	Arabica	No	A-Y
20	Santos	Arabica	No	A-N
21	Vietnam	Robusta	Yes	R-Y
22	Ethiopia	Arabica	Yes	A-Y
23	Brazil	Arabica	Yes	A-Y
24	Colombia	Arabica	Yes	A-Y
25	Santos	Arabica	No	A-N
26	Rerou	Arabica	No	A-N
27	Vietnam	Robusta	Yes	R-Y
28	India	Robusta	Yes	R-Y
29	Congo	Arabica	Yes	A-Y
30	Santos	Arabica	Yes	A-Y
31	Honduras	Arabica	No	A-N
32	Vietnam	Arabica	Yes	A-Y
33	Costa_Rica	Arabica	Yes	A-Y
34	India	Robusta	Yes	R-Y
35	Nicaragua	Arabica	Yes	A-Y
36	Brazil	Arabica	Yes	A-Y
37	Colombia	Arabica	Yes	A-Y
38	Burundi	Arabica	Yes	A-Y
39	Peru	Arabica	No	A-N
40	Mexico	Blend	No	Blend-No
41	Blend	Arabica	No	A-N
42	Rwanda/Congo	Arabica	Yes	A-Y
43	India	Arabica	Yes	A-Y
44	Brazil	Arabica	Yes	A-Y
45	Congo	Arabica	Yes	A-Y
46	Papua_NG	Arabica	Yes	A-Y
47	Brazil	Arabica	Yes	A-Y
48	Mexico	Arabica	Yes	A-Y
49	Guatemala	Arabica	Yes	A-Y
50	Vietnam	Robusta	Yes	R-Y
51	Vietnam	Robusta	No	R-N
52	Vietnam	Robusta	Yes	R-Y
53	Mexico	Robusta	Yes	R-Y
54	Peru/Honduras	Arabica	Yes	A-Y
55	Peru	Arabica	Yes	A-Y
56	Hawaii	Arabica	Yes	A-Y
57	China	Arabica	Yes	A-Y
58	Ivory_Coast	Robusta	Yes	R-Y
59	Tanzania	Robusta	Yes	R-Y
60	Vietnam	Robusta	Yes	R-Y
61	Vietnam	Arabica	Yes	A-Y
62	Honduras	Arabica	Yes	A-Y
63	Kenya	Arabica	Yes	A-Y
64	Uganda	Robusta	Yes	R-Y

Table 2
The concentrations of process contaminants (μg/kg) in roasted coffee, and the concentrations of free amino acids (mg/kg) and sugars (g/100 g) measured in dry matter of green coffee (n = 63). The compounds were sorted in descending order according to their mean (median) concentrations (expressed with a maximum of 4 significant digits).

	Compounds (notation)	Minimum (μg/kg)	Mean (median) (μg/kg)	Maximum (μg/kg)
Process contaminants	2-Methylfuran (PC2)	12,100	21,000 (21300)	27,500
	Furan (PC1)	4300	5930 (5990)	7400
	2,5-dimethylfuran (PC5)	948.4	2170 (2250)	3200
	3-Methylfuran (PC3)	574.1	1041 (1070)	1390
	Thiophene (PC7)	265.4	434.8 (419.8)	686.8
	2-methylthiophen (PC8)	147.6	226.9 (228.2)	296.9
	Acrylamide (PC9)	99.8	236.9 (204.8)	558.8
	2-ethylfuran (PC4)	87.8	189.5 (191.3)	273.2
	2,3-dimethylfuran (PC6)	38.7	84.2 (86.0)	139.7
		Minimum (g/100 g)	**Mean (median) (g/100 g)**	**Maximum (g/100 g)**
Sugars	Sucrose (S3)	2.8	8.5 (9.0)	11.9
	Glucose (S2)	0.3	0.5 (0.4)	0.9
	Fructose S1)	0.2	0.4 (0.4)	1.1
		Minimum (mg/kg)	**Mean (median) (mg/kg)**	**Maximum (mg/kg)**
Amino acids	Glutamate (AA2)	395.1	907.5 (943.3)	1493
	Asparagine (AA3)	220.8	708.7 (665.4)	2382
	Aspartate (AA1)	276.2	440.0 (436.9)	670.4
	Alanine (AA11)	141.4	315.6 (307.6)	555.7
	Serine (AA4)	108.7	201.0 (198.5)	425.6
	Proline (AA20)	109.4	183.1 (176.8)	335.5
	Phenylalanine (AA16)	44.0	159.1 (140.2)	324.7
	Arginine (AA10)	14.7	160.3 (127.0)	739.6
	Tryptophane (AA15)	ND	140.0 (122.8)	343.2
	Valine (AA13)	44.4	97.6 (96.2)	201.2
	Lysine (AA19)	ND	78.9 (76.8)	164.8
	Tyrosine (AA12)	5.5	73.9 (68.7)	177.2
	Histidine (AA6)	ND	54.3 (59.8)	112.3
	Leucine (AA18)	2.4	65.0 (59.3)	163.7
	Isoleucine (AA17)	8.0	55.9 (55.0)	110.4
	Methionine (AA14)	ND	55.9 (51.3)	257.4
	Threonine (AA8)	ND	43.0 (44.6)	104.5
	Glycine (AA7)	14.8	44.6 (39.1)	92.0
	Glutamine (AA5)	ND	36.1 (38.3)	114.3
	Citrulline (AA9)	ND	9.7 (7.6)	33.5

purchased from Chem-Lab (Zedelgem, Belgium). Ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) derivatization agents were supplied by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile HPLC grade and trichloroacetic acid (TCAA) were bought at Acros Organics (Geel, Belgium). Carrez Solution I (14% $K_4Fe(CN)_6$) and pyridine comes from Merk (Darmstadt, Germany). 3-mercaptopropionic acid and hydroxylamine hydrochloride were respectively acquired from Thermo-Fisher (Waltham, MA, USA) and UCB (Brussels, Belgium). Finally, Milli-Q water was prepared with a Millipore system from Merck.

2.2. Roasting conditions

The roasting was performed under controlled conditions to reduce the variability in the results generated by the process itself. The roasting experiment was performed using a dedicated coffee roaster (Neuhaus neotec RFB-S) that allows roasting small amount of coffee under controlled temperature and time. The roasting program consists in progressively heating 500 g of each batch from 80 °C to 230 °C during 8 min. At the end of the roasting program, the temperature of samples was quickly reduced by ventilation inside the roaster to stop the roasting process. Samples were packed in airtight coffee bags and stored in a cold chamber at 4 °C until aroma analysis. 17 samples were roasted randomly in duplicate to estimate the variability on the combined roasting-analysis workflow.

2.3. Chemical analysis

2.3.1. Dry matter content

First, the dry matter content was determined by measuring the weight loss after heating 5 g of ground green coffee samples at 105 °C using a hot air oven for 3 h. The samples were cooled to room temperature in a desiccator and weighed to the nearest 1 mg. The same procedure was repeated until a constant weight was obtained.

2.3.2. The analysis of free amino acids in green coffee beans

Free amino acids were analyzed by high performance liquid chromatography coupled to fluorescence detection (Geleta & de Meulenaer, 2019). Briefly, 2 g of ground green coffee were incubated for 10 min at room temperature with 100 mL of TCAA solution (15% v/v) with the pH adjusted at 2.2 using 10 M NaOH, Then the sample was filtered through a nitrogen-free filter (185 mm, SCHLEICHER & SCHUELL, Dassel, Germany) and 940 μL of the extract were introduced to LC-Vial with 30 μL of each internal standard (norvaline and sarcosine) at 10 nmol/μL. Primary and secondary amino acids were derivatized with OPA and FMOC solutions in the injector of an Agilent 1100 HPLC system (Agilent Technologies, Switzerland). The first solution is prepared by dissolving 100 mg of OPA and 100 mg of 3-mercaptopropionic acid in 10 mL of a 0.4 M borate buffer adjusted to pH 10.2. The second one contains 25 mg of FMOC in 10 mL of acetonitrile. Chromatographic separation was performed on a Zorbax Eclipse plus column (4.6 × 150 mm, Agilent Technologies, Palo Alto, CA, USA) with a flow rate of 1 mL/min and two mixes of solvents: "A" composed of 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7{\cdot}10H_2O$ and 5 mM NaN_3 in Milli-Q water at pH 8.2, and "B" composed of 45% acetonitrile, 45% methanol and 10% Milli Q water. Eluting conditions expressed as a portion of mobile phase "A" were 95% from 0 to 0.5 min, 43% at 20.0 min, 0% at 20.1 min until 23.5 min, and 95% at 23.6 min until 25.0 min. The detection was achieved fluorometrically with excitation and emission wavelengths equal to 340 and 450 nm respectively between 0 and 16.3 min, and 266 and 305 nm respectively between 16.3 and 25.0 min. The results were quantified on g/kg dry matter basis using internal calibration curves constructed in a range of 10–200 pmol/μL. Free cysteine could not be detected in the

samples.

2.3.3. The analysis of sugars in green coffee beans

Green coffee bean samples were analyzed for their content of sugars using gas chromatography-flame ionization detector (GC-FID) (de Wilde et al., 2005). Briefly, 10 g of ground green coffee was spiked with the phenyl β-D-glucopyranoside (6 mg/mL) as internal standard and incubated in water at 60 °C for 30 min. After cooling to room temperature, the samples were cleaned-up with Carrez I and II (5 mL each) to precipitate proteins which could interfere the later derivatization reaction. After samples filtration using 185 mm filters, 1 mL was dried under nitrogen. The residue was derivatized in two steps, first an oximation carried out at 100 °C for 30 min using 500 μL of a solution containing 2.5 g of hydroxylamine hydrochloride dissolved in 100 mL of dry pyridine, followed by derivatization at room temperature with 100 μL of HMDS and 10 μL of TFA during 10 min.

The sample was centrifuged and the upper layer was kept for analysis using a Varian 3380 GC-FID (Varian Instrument Group, Walnut Creek, CA). 1 μL was injected in the GC injector at 295 °C in split mode (1/40), the separation was performed on a 30 m × 0.25 mm × 0.25 μm VF-1 ms chromatographic column (Agilent Technologies, Palo Alto, CA, USA) and using Helium as mobile phase at 1 mL/min. The temperature program was the following, starting at 180 °C for 2 min, ramped at 10 °C/min to 290 °Cand maintained at 290 °C for 10 min. The flame ionization detector was operated with a mix of hydrogen and air at 30 and 300 mL/min, respectively. Helium at 20 mL/min as makeup gas. The detector temperature was set at 340 °C.

2.3.4. The analysis of volatile compounds in roasted coffee beans

Prior to analysis, a solution containing approximately 200 ng/mL of furan, alkylfurans, thiophene and 2-methylthiophene was prepared by introducing and weighting 10 μL of each analytical standard in a 20 mL airtight vial completely filled with methanol (this stock solution can be kept for two weeks at −20 °C). Then, a second solution was daily prepared by introducing 10 μL of this mix stock solution in a 20 mL vial filled with Milli-Q water. Internal standards solution containing the deuterated labelled furans was prepared using the same protocol.

First, coffee samples were ground and homogenized at 4 °C using Tristar Koffiemolen coffee blinder, then 50 to 100 mg of ground coffee and 1 mL of brine were introduced in a 20 mL headspace vial sealed with a PTFE/Silicone septa. Samples were then spiked through the septa with approximately 80 ng of each deuterated labelled furans standards. Vials were thoroughly vortexed at 750 rpm for 1 min prior to extraction by headspace-solid phase microextraction (HS-SPME). HS volatile extractions were performed at 20 °C during 15 min using a SPME fiber coated with 50/30 μm of DVB/CAR/PDMS (Supelco St. Louis, MO, USA). The HS-SPME was performed automatically on a CTC Combi-Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). Thermal desorption of the volatiles from the SPME fiber was carried out in the GC injector for 1 min at 230 °C. After each extraction-desorption cycle, the fiber was cleaned for 20 min at 280 °C under a gentle He flow. Fiber blanks were analyzed daily to check that no carry-over was present. Gas Chromatography separation was performed in a Trace-GC 2000 (Thermo-Scientific, Waltham, MA, USA) oven. The injection was performed in split mode using a Programmable Temperature Vaporization (PTV) injector. The chromatographic separation was achieved on a PoraBond-Q (25 m × 0.32 mm × 5 μm) column (Varian, Palo Alto, CA, USA) by elution at a constant flow rate of 1.7 mL/min He (Alpha Gas 2, Air Liquid, Belgium). The temperature program starts at 45 °C for 1 min, ramp at 120 °C/min to 120 °C, kept for 20 min, followed by a temperature ramp of 10 °C/min until 260 °C and hold for 2 min.

Detection by Mass spectrometry was performed with a PolarisQ ion-trap (Thermo-Scientific, Waltham, MA, USA). The ions were produced by a 70 eV positive Electronic Ionization (EI), with the ion source heated at 200 °C. The acquisition was recorded in full scan mode in a 35–300 m/z range. The identification of the detected molecules in samples was based on the retention time of the standards and their mass spectra. The quantification of furan compounds was carried out by isotopic dilution method within a range of (45–1800) ng/vial for 2-methylfuran and (10–900) ng/vial for the remaining furan compounds. The quantification of thiophene and 2-methylthiophene was performed through a calibration curve under a linear regression within a range of (10–500) ng/vial.

2.3.5. The analysis of acrylamide in roasted coffee beans

The analysis of acrylamide was based on the method released by Process Contaminants EU Reference Laboratory (EURL) (Granby, 2019). Briefly, 30 g of roasted coffee beans were ground using a coffee grinder, then 1 g of ground coffee was introduced in a 20 mL centrifuge tube and spiked with 600 μL of acrylamide-d_3 (1 ng/μL in Milli-Q water). After the homogenization of the mixture using a vortex, 20 mL of Milli-Q water and 1 mL of dichloromethane (DCM) were added to perform the extraction with a rotary shaker at 350 rpm for 30 min. Samples were then centrifuged for 5 min at 3500 rpm and the upper extracts were kept for clean-up. This purification was done by solid phase extraction (SPE) using Isolute multi-mode 300 mg 3 mL cartridges (Biotage, Uppsala, Sweden). Prior to the clean-up, the cartridges were conditioned with 1 mL UPLC grade acetonitrile, followed by 2 × 2 mL of Milli-Q water. Then 1500 μL of the upper extract was eluted through the SPE cartridge to "waste". And an additional 400 μL of the extract was eluted and filtered into LC-vials using 0.2 μm Acrodisc® One, PTFE filters (VWR Radnor, PA, USA).

The analysis was performed using an ACQUITY UPLC I-Class liquid chromatograph coupled to Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The chromatographic separation was achieved at 30 °C using BEH C_{18} (2.1, 150 mm, 1.7 μm) (Waters) with 0.1% formic acid in 95/5 (water/acetonitrile) at flow rate of 0.2 mL/min. The identification was based on the relative retention time and on two diagnostic transitions (72.13 m/z > 55.10 m/z and 72.13 m/z > 54.30 m/z) obtained by collision induced dissociation (CID) with Argon at 8 and 12 V respectively. The quantification was performed using isotopic dilution method with calibration curves constructed from blank sample and 5 calibration points prepared in a range between 80 and 800 ng/g of coffee using acrylamide-d_3 (daughter ion: 75.20 m/z > 58.17 m/z at 10 V) as internal standard.

2.4. Statistical analysis

The dataset used in the study consisted of 64 individual coffee samples (Table 1) with different geographical origin, type (12/64 Robusta, 51/64 Arabica, 1/64 blend) and caffeine content (55/64 caffeinated, 9/64 decaffeinated). From each sample, the concentration of nine process contaminants (PC), 20 free amino acids (AA) and three sugars (S) had been determined (Table 2). Because of missing sugar content data, sample ID34 (Table 1) was excluded from all statistical analyses.

Statistical analysis was performed in three consecutive steps, using either R 4.1.1 (R Core Team, 2021; steps 1–2) or JMP v.15 (step 3). Firstly, agglomerative HCA was performed using the function pheatmap from the package **pheatmap** (Kolde & Kolde, 2015). Analyses were performed with both non-standardized (measured values) and standardized (z-scores) variables, Euclidean distance and average linkage. Secondly, Spearman correlation analysis was performed using the function rcorr() from the package **Hmisc** (Harrell & Harrell, 2019) and visualized with the package **corrplot** (Wei & Simko, 2021). All variables were retained in non-standardized form. Finally, partial least squares regression (PLS) was applied in accordance with the protocol of Kuuliala et al. (Kuuliala et al., 2018). Briefly, AAs and sugars were used as predictor variables and a given process contaminant as the response variable. Models were generated using standardized variables, (z-scores), the NIPALS algorithm and leave-one-out cross validation. The number of factors was chosen to minimize the root mean predicted residual sum of

squares (PRESS). Most relevant constituents were identified by the following three criteria: 1) positive correlation with the response variable, 2) variable importance in projection (VIP) $\geq$ 1, 3) positive regression coefficient.

3. Results

3.1. *The levels of amino acids, sugars, and process contaminants in green or roasted coffee beans*

The concentrations of the nine food process contaminants (PC1-9), twenty amino acids (AA1-20) and three sugars (S1-3) were measured in 64 samples of green and roasted coffee beans (see the complete list of coffee batches in Table 1). Amino acids and sugars were quantified in green beans, while food process contaminants were determined in the roasted beans. Table 2 summarizes the mean (median), minimum and maximum levels for each of the 32 compounds analyzed. The concentrations of process contaminants are expressed as µg/kg in roasted coffee, and the concentrations of free amino acids as mg/kg and sugars as g/100 g measured in green coffee dry matter, respectively. The compounds were classified in descending order according to their mean (median) concentration. All individual levels for the 64 samples analyzed are provided in the supplementary information in Table S1. Levels and comparison with the literature are further discussed in Section 4.

Pending upon the particular process contaminant considered, relative standard deviations amounted between 7% for furan and 13 % for 2-methylthiophene for the process contaminants analyzed via gas chromatography (GC), while for acrylamide a relative standard deviation of 10 % was obtained. These data indicate that the combined workflow of roasting and quantitative analyze of the considered process

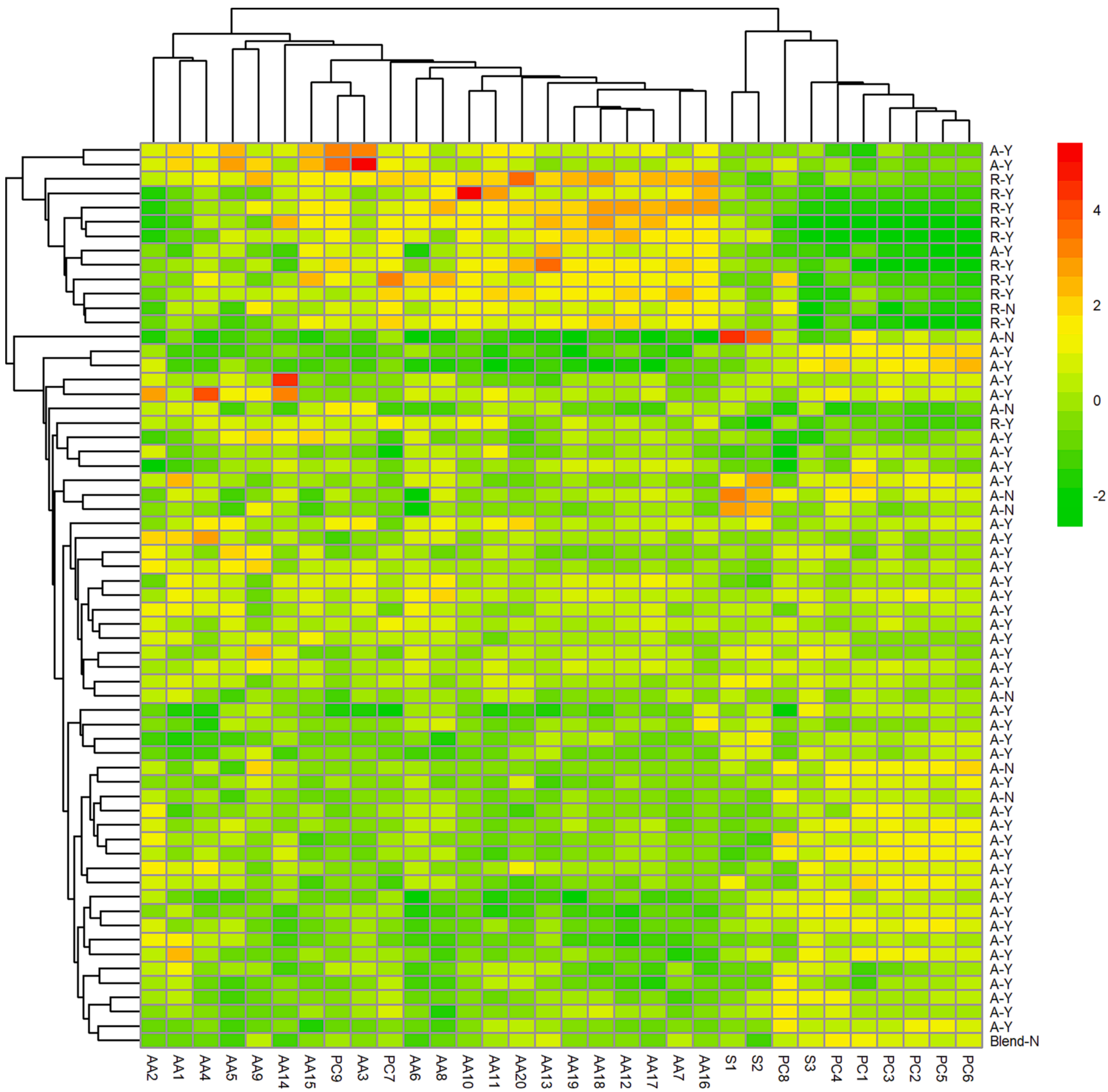

Fig. 1. Clustering results: standardized variables. Individual samples (rows) are labelled as X-Y, where X indicates the type (A = Arabica, R = Robusta) and Y the caffeine content (Y = Yes, N = No). For variable codes, see Table 2.

contaminants was repeatable.

3.2. Clustering

The HCA results are presented in Fig. 1 and Supplementary Fig. S1, showing the clustering of both individual coffee samples (rows) and variables (columns). When using non-standardized data (Supplementary Fig. S1), distinct sub-clusters of coffee samples could be observed. However, due to the differences in concentration magnitudes and units (Table 2), clustering of variables demonstrated the difference between 2-methylfuran (PC2) and the other studied process contaminants but otherwise provided little information. Consequently, standardization was applied in order to assess the similarity irrespectively of the concentration magnitude and/or unit (Fig. 1).

In Fig. 1, two main clusters could be attributed to the type of coffee. The first cluster was largely comprised of Robusta samples, whereas the second cluster mainly contained Arabica samples. Notably, both the sugar and furan contents were generally lower in Robusta when compared to Arabica, whereas the contents of amino acid and acrylamide were typically higher. No clear trends related to the geographical origin or decaffeination process were detected and are thus not examined further in the present study. On the other hand, when considering the studied variables, a separation between the amino acids and the other chemical constituents could be observed. Two major clusters were formed: the first cluster contained the sugars and most of the process contaminants, while the second cluster contained the amino acids. The majority of the variables in the first cluster were also clustered together at lower heights than those in the second cluster, indicating a higher overall similarity among PC/S compounds (particularly PC1-6 and S3) than among the amino acids. Consequently, the correlation between the variables is examined further in Section 3.3.

3.3. Correlation analysis

The results of the correlation analysis are shown in Fig. 2. Significant correlation ($p < 0.05$) is indicated with an asterisk (*), while the correlation coefficient (ρ) is displayed on a color scale where -1 indicates perfect negative correlation and $+1$ perfect positive correlation. In line with the clustering results (Section 3.2), significant positive correlation was observed 1) among furans (PC1-6), 2) among most of the amino acids, and 3) between sucrose content (S3) and the furans, whereas significant negative correlation was observed between several amino acids and furans. Glutamic acid (AA2) was the only amino acid that showed positive correlation with furan compounds, particularly with 3-methylfuran (PC3) ($\rho = 0.56$). Thiophene (PC7) showed negative correlation with the furans and positive correlation with most amino acids. However, insignificant correlation was found between thiophene and sulfur-containing methionine (AA14) as well as 2-methylthiophene (PC8) and other studied variables. Acrylamide (PC9) content was found negatively correlated with furans and sucrose, while showing

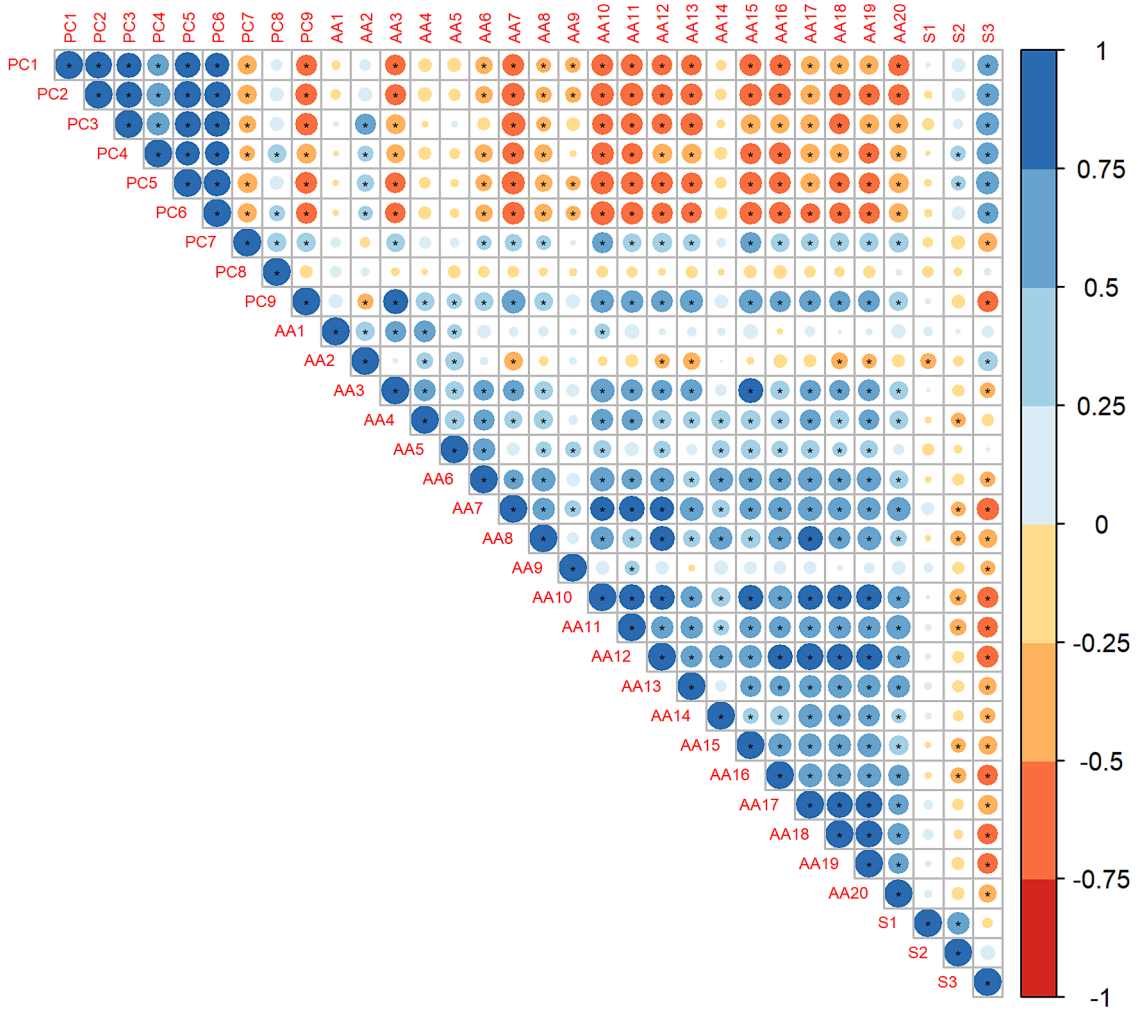

Fig. 2. Correlation analysis results. Significant correlation ($p < 0.05$) is indicated with an asterisk. Spearman correlation coefficients are shown on a color scale, where blue represents positive and red negative correlation. For variable codes, see Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strong positive correlation ($\rho = 0.78$) with asparagine (AA3). Finally, fructose (S1) and glucose (S2) showed weak or insignificant correlation with the other studied variables. However, it should be noted that their concentrations were small (mean concentration $\leq$ 0.5 g/100 g) compared to the dominating sugar, i.e., sucrose (S3; mean concentration 8.5 g/100 g). Based on all these results, 2-methylfuran (PC2) and 3-methylfuran (PC3) were selected for further investigation by PLS regression (Section 3.4).

3.4. Partial least squares (PLS) regression

The PLS results are shown in Fig. 3. The number of factors that minimized the root mean PRESS was 4 and 3 for models with 2-methylfuran (PC2) and 3-methylfuran (PC3) as the y-response, respectively. The first two factors covered 68.9 and 75.5 % of the y-response variance. Overall, few chemical constituents fulfilled any of the defined selection criteria (Section 2.4). Most of the studied amino acids were not only negatively and/or weakly correlated with the studied process contaminant, but also remained below the VIP threshold. Only sucrose (S3: both PC2 and PC3) and glutamic acid (AA2: PC3) fulfilled all three selection criteria.

4. Discussion

According to the literature on sugar concentrations in green coffee, sucrose is expected to be found between 3.0 and 9.0 % (or g/100 g) of dry matter, while glucose and fructose are expected to be more than an order of magnitude lower, i.e. between 0.2 and 0.5 % (Baig & Keservani, 2016; Wei & Tanokura, 2015). In Table 2, the mean (median) concentration of sucrose is 8.5 % (9.0 %), while much lower levels of glucose and fructose (0.4 %) were reported. One should note that mean sucrose level in Arabica (9.3%) is twice higher than in Robusta (4.6%, raw data in Table S1). These results are in agreement and confirm the concentrations already reported for market coffee.

For free amino acids, the highest concentrations in the literature were reported for glutamate (0.13 %) followed by asparagine, alanine and aspartate (0.05 % for each), while the remaining amino acids were lower than 0.02 % (Baig & Keservani, 2016; Wei & Tanokura, 2015). The concentrations of free amino acids shown in Table 2 are expressed in mg/kg. After conversion to percentages for comparison with other works, the highest median concentrations were measured for glutamate (0.094 %), followed by asparagine, aspartate and alanine with median concentrations of 0.067, 0.044 and 0.031 %, respectively. The remaining amino acids were $<$ 0.02 %. In addition, the mean total content of free amino acids measured in this study are significantly higher in Robusta (0.46%) compared to Arabica (0.37%).

With regard to the process contaminants measured in this work, the highest mean concentrations were measured for 2-methylfuran (21000 µg/kg), followed by furan, 2,5-dimethylfuran and 3-methylfuran in the range between 5930 and 1041 µg/kg. These values are consistent with previously reported values (EFSA, 2017; Kettlitz et al., 2019; Alsafra et al., 2022). The median concentrations of other alkylfurans, thiophenes and acrylamide are all generally below 500 µg/kg. The median concentration of acrylamide in roasted coffee reported by (Arisseto et al., 2007; EFSA, 2015) is 174 µg/kg, which is consistent with the median found in this study (i.e. 205 µg/kg).

In the present study, different statistical methods were used for characterizing the chemical composition of the studied coffee samples. As the first exploratory step, hierarchical cluster analysis was applied. HCA allowed the comparison of different coffee samples or variables (Fig. 1), such as pointing out major differences in both precursor and process contaminant levels between Robusta and Arabica samples.

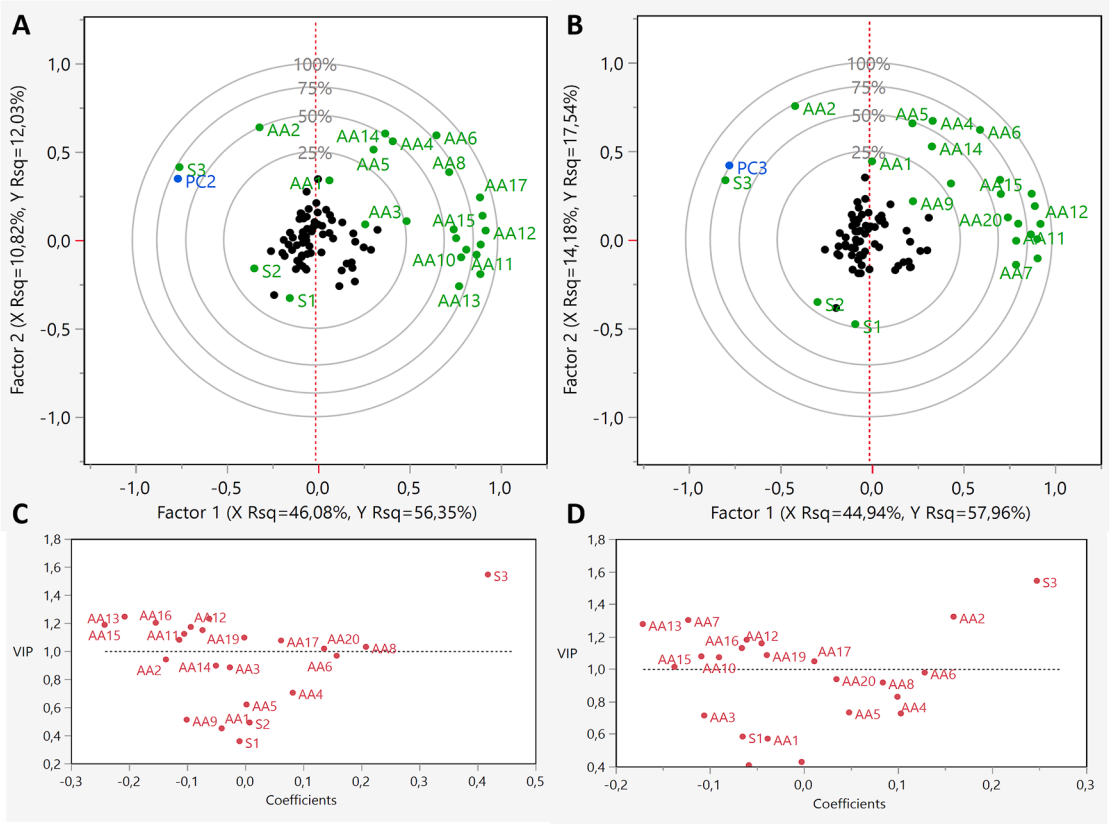

Fig. 3. Partial least squares (PLS) regression results: biplots (A-B) and variable importance in projection (VIP) vs. regression coefficient plots (C-D). Either 2-methylfuran (PC2: A and C) or 3-methylfuran (PC3: B and D) is treated as the response variable, chemical constituents (amino acids and sugars) as the predictor variables and individual coffee samples (n = 63) as samples. For variable codes, see Table 2.

However, no clear trends related to geographical origin or decaffeination could be observed in the present study. It should be noted that the samples' geographical diversity was relatively high and the number of decaffeinated samples low (9/64) when compared to the total number of samples. Further data collection would thus be needed to assess potential differences due to geographical location and/or caffeine content.

Secondly, while it should be noted that correlation does not equal causality, the results of the correlation analysis (Fig. 2) could be used to formulate some hypothesis with respect to the complex chemical phenomena leading to the formation of process contaminants during the roasting of coffee beans. For example, positive correlation was found in the present study between glutamic acid (AA2) and 3-methylfuran. Interestingly, Wu et al. (Wu et al., 2018) reported that glutamic acid hydrochloride was a very efficient catalyst to convert fructose to 5-hydroxymethylfurfuraldehyde, which is a key intermediate in sugar degradation reactions. As indicated in the introduction, sugar degradation reactions have been identified as an important route to the formation of furan. On the other hand, when it comes to the formation of thiophene and 2-methylthiophene, limited reports are still available in the scientific literature.

Finally, PLS was used for identifying potential predictors for the production of 2-methylfuran and 3-methylfuran in roasted coffee beans (Fig. 3). The obtained results suggest a strong link with the presence of sucrose. In contrast to these results, however, Limacher et al. (2008) previously showed that the formation of 2-methylfuran from different reducing sugars remained rather restricted, especially when compared to the formation of furan (Limacher et al., 2008). It was enhanced in the presence of particular amino acids (respectively threonine, phenylalanine and serine). In our study, no positive correlation between these amino acids and the content of methylfurans in general and 2-methylfuran in particular could be observed. It thus remains remarkable that in coffee 2-methylfuran is produced much more than furan, while in model systems studying the role of sugar degradation in the formation of furan and 2-methylfuran especially furan is formed. Although the scientific evidence linking sugar degradation to the formation of 2-methylfuran is not strong, the current study does suggest such a potential link.

Overall, the obtained results suggest that sucrose is the key precursor for the formation of furan and methylfurans during coffee roasting. Although there is a general agreement that sucrose as such is not reactive, upon roasting it is hydrolyzed to fructose and glucose, which are unstable and prone to dehydration and fragmentation reactions, leading to the formation of a variety of reactive carbonyl substances which further on will react to form furan and to a lesser extent methylfurans (Limacher et al., 2008). Furthermore, this study supports the importance of asparagine as a key determinant in the formation of acrylamide. Already in 2002, Mottram et al and Stadler et al. independently showed that asparagine is the carbon source of acrylamide and was able to form it during the Maillard reaction (Mottram et al., 2002; Stadler et al., 2002). Bagdonaite et al. (2008) showed that asparagine is the limiting factor for acrylamide formation in coffee, albeit on basis of a much more limited dataset (Bagdonaite et al., 2008). Indeed, given the excess of sucrose and that the precursor of fructose and glucose are generated in situ (Bagdonaite et al., 2008), these authors found a positive correlation between the sucrose content of green beans and the acrylamide content after roasting. In this study, Robusta samples proved to be more prone to acrylamide formation (mean level of 329 μg/kg) compared to Arabica samples (mean level of 217 μg/kg). The mean values were calculated from raw data available in supplementary information Table S1.

Furthermore, the outcomes of the present study also support earlier findings that furan and acrylamide seem to be inversely related (Lachenmeier et al., 2019). This has been typically attributed to the difference in the degree of roasting, i.e. in a lighter roasted coffee more acrylamide is typically found, while in dark roasted coffee, furan and related compounds dominated. It could be speculated that in the initial phase of the roasting process, sufficient amounts of water are still present, thus enabling the reactants involved in the Maillard reaction (i.e. reducing sugars and amino acids) to react with each other, as the molecular mobility is still sufficiently high. These reactions will lead in the presence of asparagine to the formation of acrylamide. In addition, sugar degradation is facilitated by the availability of amino acids, leading to the formation of furan and potentially methylfurans. In the later stages of the roasting process, the availability of water is reduced and thus a shift in the reactions is obtained in favor of reactions not requiring two or more molecules to react. As such, degradation reactions of sugars are favored as these do not require the presence of other reactants. However, given that the roasting conditions were the same for all the samples in the present study, another mechanism should be involved explaining the inverse relationship between furan/methylfurans and acrylamide. In view of the negative correlation between the content of most free amino acids (except glutamic acid) in the green beans and the furan and methylfuran content in the roasted beans, it is hypothesized that if the pool of free amino acids is large enough, Maillard type of reactions will be favored as long as the mobility of the reactants allows it. The reactive sugar fragmentation products will react with free amino acids, creating a sink for reactive carbonyls which are in fact pulled away from reactions leading to the formation of furan and methylfurans. During roasting however, the free amino acids become depleted as shown by (Bertuzzi et al., 2020). Once the pool of free amino acids becomes depleted, sugar fragmentation products are likely to condense preferably with themselves, leading to the formation of furan and methylfurans.

5. Conclusion

The present study provides new insights into examining the formation of furans, methylfurans, thiophenes and acrylamide during the roasting of over 64 different coffee varieties procured from over the globe. By applying controlled roasting conditions, samples could be compared irrespective of the effect of roasting time and duration. Multivariate statistical analysis allowed to identify promising relationships between process contaminants and their potential precursors. Overall, the outcomes of the study extend the current understanding of the formation of aforementioned process contaminants in coffee. Sucrose was identified as the primary precursor for the formation furan and methylfurans, while asparagine was identified as the principal precursor for acrylamide. Coffee samples with a lower content of free amino acids generated more furans and methylfurans. In addition, this study provides interesting directions for further research efforts. In particular, the potential role of glutamic acid in favoring the degradation of reducing sugars and thus the formation of furans as well as the inverse relation between furan and acrylamide would deserve further attention.

CRediT authorship contribution statement

Zouheir Alsafra: Investigation, Validation, Writing – original draft. **Lotta Kuuliala:** Formal analysis, Writing – original draft. **Georges Scholl:** Writing – review & editing, Visualization. **Claude Saegerman:** Writing – review & editing. **Gauthier Eppe:** Conceptualization, Methodology, Resources, Supervision, Funding acquisition. **Bruno De Meulenaer:** Conceptualization, Methodology, Resources, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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The authors contributed equally to this study: ZA carried out the roasting experiments and the chemical analysis on the samples. LK carried out the statistical analysis of the data. LK acknowledges the support of the Research Foundation Flanders (FWO) for a junior post-doctoral fellow (1222020 N). Roasting experiments were carried out at Beyers and samples were collected by various coffee producers and traders. Sample collection was coordinated by KOFFIECAFE. The authors would like to thank An Maes and Margot Vansteenland from UGent for their active collaboration in samples analysis.

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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.2023.136655.

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