ISBN: 978-628-95207-8-1. ISSN: 2414-6390. Digital Object Identifier: 10.18687/LACCEI2024.1.1.1711

In-house validation of analytical methods for the determination of NFCs in roasted coffee and cocoa

Medina-Orjuela, Maria-Elena[1], Barrios-Rodríguez, Yeison-Fernando[1,2], Carranza Gutierrez, Carlos Edwin[3], Gutierrez-Guzman, Nelson[1], Amorocho-Cruz, Claudia-Milena[1], Girón-Hernández, Joel[4]

[1]Centro Surcolombiano de Investigación en Café (CESURCAFÉ), Universidad Surcolombiana, Av. Pastrana Borrero Carera 1, 410001. Neiva, Colombia, medinao.mariae@gmail.com, maria.medina@usco.edu.co

[2]i-Food, Instituto Universitario de Ingeniería de Alimentos FoodUPV, Universitat Politècnica de València, Camino de Vera s/n, 46021 Valencia, Spain, yfbarrod@upv.es, yeison.barrios@usco.edu.co

[3]Escuela de ciencias agrícolas, pecuarias y del medio ambiente, Universidad Nacional Abierta a Distancia, Bogotá, Colombia, carlos.carranza@unad.edu.co

[4] Department of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University, NE1 8ST Newcastle Upon Tyne, joel.l.g.hernandez@northumbria.ac.uk

Abstract

Process contaminants (NFCs) such as acrylamide, furfuryl alcohol (FFA), and hydroxymethylfurfural (HMF) have been linked to the development of adenomas, toxicity, and carcinogenicity. These NFCs are found in roasted coffee and cocoa consumed worldwide. Therefore, developing and evaluating reliable analytical methods for their determination is imperative. These methods are mainly based on chromatography, mass spectrometry, and absorbance detectors. However, information on the validation of analytical methods for determining NFCs, particularly in cocoa, is scarce, leading to uncertainty about the validity of the results. The performance parameters of the methods provide the reliability required for their intended use. Therefore, this study validated the performance parameters of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and Liquid Chromatography coupled with a Diode Array Detector (LC-DAD) methods for determining NFC in roasted coffee and cocoa to provide reliable methods. The methods evaluated demonstrated a satisfactory fit to the linear model, with a coefficient of determination (R^2) greater than 0.997. The accuracy of the methods ranged between 80 and 120%, and the precision and intermediate precision were below 9% RSD and below 16%. The reproducibility of the methods was between 0.36 and 2% in terms of HorRat. The methods were considered suitable for the determination of NFCs in high consumption matrices such as cocoa and coffee, in accordance with international guidelines of the Food and Drug Administration (FDA) and the Association of Official Analytical Chemists (AOAC). Obtaining these performance indicators for determining compounds of high interest, such as acrylamide, contributes to remedying the lack of information on the validation of methods for quantification, especially in cocoa. In addition, the importance of using salts for extract clean-up was established, which consistently and reliably eliminates interferences, allowing for greater sensitivity of the methods.

Keywords

validation, figure of merit, hydroxymethylfurfural, furfuryl alcohol, coffee, cocoa.

I. Introduction

Digital Object Identifier: (only for full papers, inserted by LACCEI).
ISSN, ISBN: (to be inserted by LACCEI).
DO NOT REMOVE

Process contaminants or neoformed compounds (NFCs) such as acrylamide, furfuryl alcohol (FFA), and hydroxymethylfurfural (HMF) are compounds that can have adverse health effects. They are formed in foods such as coffee and cocoa when subjected to at high temperatures (<120 °C) through various chemical reactions, including the Maillard reaction, caramelization of sugars (pyrolysis), Strecker degradation, and lipid oxidation. [1].

Acrylamide is an organic molecule whose main formation pathway is based on the interaction between the carbonyl group of free sugars and the amino group, also known as the Maillard reaction. A highly reactive molecule is absorbed through the gastrointestinal tract, metabolized by conjugation with glutathione and epoxidation to glycidamide, and distributed throughout the organs, capable of ionic binding and free radicals [2]. The chemical properties of this molecule have been studied, and adverse effects have been observed in rats and mice as a possible inducer of genotoxicity and potential carcinogenicity by oral ingestion in mice [3]. It was identified by the International Agency for Research on Cancer (IARC) in 1994 as possibly carcinogenic to humans (Group 2A) [1]. On the other hand, furfuryl alcohol can be formed through the Maillard reaction, degradation of sugars and polysaccharides, it is absorbed and metabolized by the human body through oxidation to furfuroic acid. Entities like the IARC have classified furfuryl alcohol in Group 2B as possibly carcinogenic. On the other hand, HMF has been the subject of research, finding mutagenic effects in the presence of the cytosolic fraction of rat liver with 3'-phosphoadenosine-5'-phosphosulfate as a cofactor of Sulfotransferase (SULT), which is also present in the human body [4]. The importance of these compounds for health and their presence in food makes it necessary to establish reliable methods for the determination of NFCs, which can be applied internally or outsourced, with the objective of enabling the control of the roasting process and the chemical quality of the products, as well as the development and research of techniques for the reduction or mitigation of NFCs in the industries.[5], [6], [7].

Liquid chromatography (LC) and gas chromatography (GC) are the most widely used techniques for the determination of these compounds due to their high sensitivity, effective separation, and simultaneous quantification of a wide variety of compounds, such as furans (HMF and FFA). The detectors associated with LC are based on the properties of matter, such as absorbance, refractive index, fluorescence, and fragmentation pattern [11]. On the other hand, GC-associated detectors are based on the determination of volatile and thermostable compounds, generally quantified or identified by their fragmentation pattern, ability to ionize and generate electric current, as well as their affinity for electrons in an electric field, which allows their capture and indirect quantification [12]. Acrylamide is usually quantified by liquid chromatography (LC) or gas chromatography (GC) and is often associated with mass spectroscopy (MS) [13], [14], [15], [16]. Meanwhile, diode arrays or ultraviolet detectors are often used to detect FFA and HMF [8].

To ensure accurate results during analyte extraction, it is necessary to perform cleaning and clarification steps on the extracts to remove possible interferences, such as proteins, fibers, and oils. These interferences, at high concentrations, can saturate the chromatographic system, which could result in mass-coupled systems in incomplete ionizations and an excessive occupation of the separation sites on the column. In addition, it can lead to poor recoveries due to the change of polarity between molecules, which limits the access of the solvent to all compounds. Also, there is a risk of co-extracting related compounds, which could increase the instrumental response and cause inappropriate or inaccurate detections, known as the matrix effect. Also, the trace composition in the extract may promote the formation/degradation of NFCs in the food during the extraction process, mainly when carried out at high temperatures [9]. One way to mitigate the inaccuracy problem is by adding internal standards, which allow the retention or signal to be corrected for concentration [17].

All this generates an uncertainty associated with the results obtained by analytical methods, which can be reduced by validating their application. Generally, this process involves the evaluation of parameters such as selectivity, linearity, limits of detection and quantification, accuracy, precision, intermediate precision, and reproducibility [18]. These data are essential to confirm the method's suitability in the samples evaluated, covering different concentration ranges, providing more excellent reliability, and ensuring the quality of the determinations made. Although this process is essential, there is a lack of information in the different scientific papers regarding the validation of analytical methods, especially for quantifying NFCs in cocoa. For this reason, the objective of the present work was to establish a methodology for the internal validation of methods for determining NFCs in coffee and cocoa by LC-MS/MS (acrylamide) and LC-DAD (HMF and FA).

II. Experimental section

A. Reagents

The reagents methanol LC-MS (≥99.97%), acetonitrile LC-MS (≥99.9%), and formic acid (≥97.5%), Carrez Clarification Kit were purchased from Merck Group, Germany. While acrylamide HPLC grade (99.8%), acrylamide deuterated 3 (d3) analytical grade (500 mg Kg-1), furfuryl alcohol analytical standard grade (99.8%), and hydroxymethylfurfural analytical standard grade (99.7%), and sodium chloride (≥99%) were purchased with Sigma Aldrich, USA. On the other hand, the QuEChERS AOAC Method Kit, composed of 400 mg Primary and secondary amine (PSA) and 1200 mg anhydrous magnesium sulfate ($MgSO_4$) was purchased from Thermo Fisher Scientific, USA.

B. Samples

The samples analyzed were roasted Arabica coffee (100 g) and a blend of roasted Trinitario and Criollo cocoa (100 g). The samples were screened for a particle size between 400 and 500 µm.

C. Obtaining extracts

Coffee and cocoa extracts for acrylamide determination

The extract was prepared at a concentration of 100 mg mL^{-1}, taking 0.5 g of ground coffee or cocoa in 4 mL of water. Then, 1 mL of acrylamide-d3 (1000 µg L^{-1}) was added and homogenized before the addition of dichloromethane in the same proportion of the solvent (5 mL). It was shaken for 5 min at 2500 rpm between each step. Next, it was centrifuged at 7000 g for 5 min; then, the aqueous phase was removed and mixed with acetonitrile and 2 g of sodium chloride (NaCl) to drive the change of the analyte to the organic phase. Subsequently, 4 mL of the organic phase were added to the 15 mL falcon tubes of the QuEChERS AOAC 2007 QuEChERS Kit, which consisted of 1200 g MgSO4 and 400 mg PSA (Thermo Scientific, USA). Finally, filtration at 0.2 µm with nylon was performed, and the samples were transferred to vials.

Coffee and cocoa extracts for FFA and HMF determination

The extracts were prepared at 40000 and 10000 mg L^{-1}, taking 50 and 200 mg, for HMF and FFA of ground coffee or cocoa in 5 mL of water, respectively. They were clarified by the addition of 0.75 ml of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]$ x $3H_2O$) at 0.15 g ml^{-1} (Carrez I) and zinc sulfate heptahydrate ($ZnSO_4$ x $7H_2O$) at 0.3 g ml^{-1} (Carrez II). After adding these substances, it was shaken for 1 minute and allowed to stand for 5 minutes. It was then centrifuged at 4500 g for 20 min and passed into a vial through 0.2 µm nylon filters [8].

Obtaining acrylamide standards

Concentrations in the range of 10 µg L^{-1} to 400 µg L^{-1} of acrylamide were performed, with the addition of 100 µL of a

1000 µg L^{-1} acrylamide-d3 solution in type I water as a standard control in both matrices.

Obtaining HMF and FFA standards

The calibration curve was made in the concentration range of 1-30 µg mL^{-1} of a mixed standard of 2-FFA, 5-HMF for two matrices, which were diluted in type I water, followed by ultrasound for 10 min, and passed through a nylon filter with a pore size of 0.22 µm.

D. Optimization of analytical methods

Optimization of the method for acrylamide determination by LC-MS/MS

A 500 µg L^{-1} acrylamide and acrylamide-d3 standard prepared in acetonitrile with 0.1% formic acid was infused into the triple quadrupole analyzer for optimization. Primarily, the capillary voltage was established by successive comparisons in the intensity of the transitions, increasing by 0.50 kV, similar to the cone and collision voltage at every 2 V.

On the other hand, the retention of acrylamide in the columns UPLC BEH C18, 130Å, 1.7 µm, 2.1 mm X 50 mm and ACQUITY UPLC HSS T3, 100 Å, 1.8 µm, 2.1 mm x 100 mm, in the composition 90% water and 10% acetonitrile both acidified with 0.1% formic acid, was compared to select the column that retained acrylamide longer.

Optimization of the method for the determination of FFA and HMF by LC-DAD.

External standards of FFA and HMF prepared at 35 mg L-1 were injected, and the absorbance of the NFCs was confirmed at 217 nm for FFA and 284 nm for HMF. The samples were then analyzed to determine the analytes' concentration and establish the linearity ranges and the solvent extract ratio. Increasing the extract ratio four times in cocoa was necessary due to the low presence of FFA.

E. Conditions for the determination of NFCs

Acrylamide quantification (LC-MS/MS)

Acrylamide was determined using Ultra Performance Liquid Chromatography-tandem mass spectrometry (UPLC-MS/MS) on an ACQUITY UPLC-I-Class system coupled to a Xevo TQ-S micro triple quadrupole mass analyzer (MS/MS) (Waters Corporation, United States). A 4 µL injection volume and a 0.2 mL min^{-1} flow rate were employed, with an Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 µm) at 30°C column temperature. The mobile phase comprised a mixture of 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B). The composition varied from an initial 90% of A to 1.5 minutes, then decreased to 50% until 1.8 minutes. Subsequently, it remained constant for 0.3 minutes, followed by a gradual increase in the percentage of B to 95% at 2.5 minutes, then decreased to 50% until 2.8 minutes. Finally, it decreased to 10% at 3.2 minutes and remained constant until 7 minutes.

The MS/MS was configured in positive electrospray ionization mode, utilizing multiple reaction monitoring (MRM) acquisition. A capillary voltage of 0.5 kV and a cone voltage of 20 V was applied, with source and desolvation temperatures set at 150 °C and 500 °C, respectively. Nitrogen gas flows for desolvation and collision were maintained at 1000 L h^{-1} and 50 L/h. The molecular ion [M+H]+ for acrylamide was monitored at m/z 72.8616 > 44.9627, 55.8925, with cone and collision voltages set at 20 V, 8 V, and 14 V, respectively. For acrylamide-d3, the [M+H]+ ion was monitored at m/z 75.0000 > 32.9900, 58.1000, with a cone voltage of 24 V and collision voltages of 8 V and 10 V, respectively [21], [22], [23].

FFA and HMF quantification (LC-DAD)

FFA and HMF were determined using High-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) on an Agilent 1200 series HPLC system (Agilent Technologies, Germany) with a PROTECOL C8 H column (150 mm X 4.6 mm, 125 µm, Machery-Nagel, Germany).

The mobile phase was composed of 80% acetic acid 0.1% v v^{-1} in type I water (A) and 20% methanol (B), with a total run time of 15 minutes, an injection volume of 20 µL, a flow rate of 1 mL min^{-1} flow rate, and a column temperature of 30 °C. The FFA compound was detected at 217 nm and 5-HMF at 284 nm [8].

F. Figure of merit

The performance parameters of the analytical methods were validated within the context of "The fitness for the purpose of analytical methods: a laboratory guide to method validation and related topics"[19], Appendix F: “Guidelines for Standard Method Performance Requirements” and “Laboratory Manual Volumen II Methods, Method Verification and Validation Sections” de la Food and Drug Administration (FDA) Office of Regulatory Affairs (ORA) [20].

Selectivity

The selectivity test was performed by comparing chromatograms of six samples of each matrix with the target analyte, six injections of the standard of the same concentration level (400 µg Kg^{-1} for acrylamide, and 30 mg Kg^{-1} for FFA y HMF), and two injections of the extraction solvent as the blank. Retention times and areas of the standards were compared. The system's reproducibility as a function of time and areas under the curve were evaluated in %RSD (Relative Standard Deviation), which was accepted when it was less than 5% [20]. Additionally, the theoretical plates (>10000) and the asymmetry factor (0.8-1.3) were evaluated as an indication of the system's suitability.

Linearity

It was evaluated by quantification in triplicate of seven concentration levels standards 5, 10, 15, 30, 70, 140, 280 y 400 µg Kg^{-1} for acrylamide, 1, 5, 10, 15, 20, 25 y 30 mg Kg^{-1} for FFA and HMF, respectively). The linearity of the system was

determined by external standard for the FFA and HMF and by internal standard for the acrylamide; the data were mathematically modeled (1), where α is the intercept, β is the slope, and *y* was the instrumental response [19].

$$y = \alpha + \beta x \quad (1)$$

Limit of Detection (LOD)and Limit of Quantification (LOQ)

The LOD and LOQ were estimated using the equation (2) and (3), respectively, where the RSD corresponds to the relative standard deviation in the intercept values [19].

$$\text{LOD} = 3 * \frac{\text{RSD}}{\beta} \quad (2)$$

$$\text{LOQ} = 10 * \frac{\text{RSD}}{\beta} \quad (3)$$

Accuracy

This parameter was quantified using the equation (4), where C_I is the instrumental analytical concentration and C_T is the theoretical concentration. Three replicates were prepared for three concentration levels and injected in duplicate (2, 8, 32 µg Kg^{-1} for acrylamide in coffee and 10, 40, 120 µg Kg^{-1} in cocoa roasted, 1, 5 y 10 mg Kg^{-1} for FFA and HMF, respectively in both food). The acceptance criterion was based on the mean percentage recovery at each level, which ranged from 80 to 110%, and the %RSD that was less than ≤ 5.0% at each percentage recovery level. In addition, the t Student statistic was applied to the averages at each level, with an acceptance value of 95% [20].

$$\text{Recovery} = \frac{C_I}{C_T} \quad (4)$$

Precision

This parameter was validated by evaluating six coffee extracts and six cocoa extracts, according to the methods described for each type of technology, which were injected only once. The acceptance criterion was less than 16% relative standard deviation and a recovery percentage between 80-120% [20].

Intermediate precision

To evaluate this parameter, two analysts were used for four consecutive days; the number of samples and injections per replicate each day was equal to those of the precision parameter. The RSD between samples from the same analyst per day was accepted when it was less than 16% [18].

Reproducibility

Reproducibility was estimated according to the HorRat equation (5). RSDri is the relative standard deviation for analyses applied on different days, and PRSDr is the predicted relative standard deviation, corresponding to 16% [18].

$$\text{HorRat} = \frac{\text{RSDri}}{\text{PRSDr}} \quad (5)$$

III. Statistical Analysis

System suitability, linearity, LOD, LOQ, accuracy, precision, intermediate precision, and reproducibility were analyzed by comparison of means and %RSD. Also, linearity was evaluated by the coefficient of determination, and model evaluation was performed using the F statistic t-test and residuals.

IV. Results and Discuss

A. *Method optimization for determining acrylamide by LC-MS/MS and determining FFA and HMF by LC-DAD.*

A comparison of the retention time of acrylamide obtained by the two columns showed a difference of 0.3 min, higher for the HSS-T3 column, and reproducibility of less than 0.4%. This may be associated with a lower carbon composition in the HSS-T3 column, conferring lower hydrophobicity, although not significantly. The flow rate of 0.3 ml min^{-1} allowed the separation of extracted compounds from analytes not retained by the column and reduced the asymmetry of acrylamide and acrylamide-d3 (Fig 1).

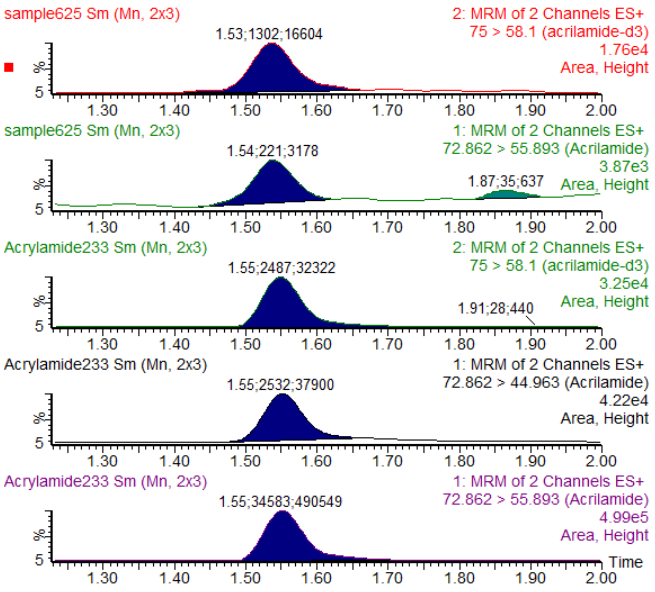

Fig 1. Chromatogram of the acrylamide standard at 280 µg Kg-1 and a roasted coffee sample chromatogram. Transitions of higher abundance of acrylamide 72.862>55.893 and acrylamide d3 75.1>58.1. Retention times 1.55 (acrylamide) and 1.54 (acrylamide-d3).

The extraction of acrylamide was performed in water because acrylamide is a small and polar molecule due to the presence of the amide group (-$CONH_2$), which enables

electrostatic interaction. This confers a good solubility in water at 20 °C [24]. The cleaning techniques were fundamental to optimize the process and increase the selectivity and sensitivity of the method. The addition of sodium chloride to the acetonitrile in the change from aqueous to organic phase was implemented to decrease the solvation of the compounds by the solvent and increase the electrostatic or coordination interactions between salt ions and acrylamide to increase the solubility of the analyte [25]. Two compounds were also selected for the second cleaning step, MgSO4 and PSA, to adsorb polar impurities from the sample and remove traces of water and other compounds from the coffee and cocoa. In addition, agitation was performed to ensure homogenization of the sample in both the solid and liquid phases, allowing clarification and concentration of acrylamide in the extract [10].

As for determining FFA and HMF by LC-DAD, the highest intensity was confirmed at 217 nm for FFA and 284 nm for HMF. The flow and gradient conditions provided good resolution and symmetry (Fig 2). FFA and HMA compounds can accept or donate electrons due to the aldehyde functional group in both compounds and the hydroxyl group in FFA. In this sense, water was selected as the extract solvent due to the asymmetric electronegative charge distribution, which confers the ability to form dipoles with water. This facilitates their interaction and solubilization in the aqueous medium, which favors their extraction and subsequent analysis.

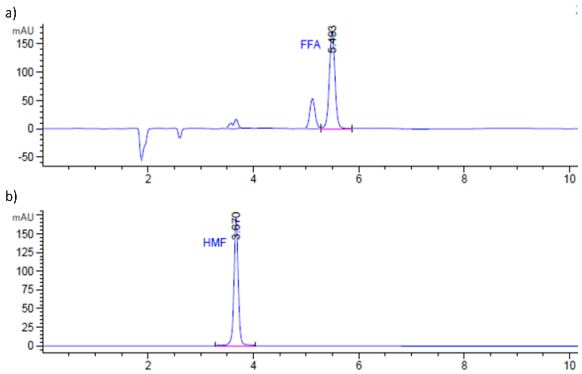

Fig 2. Chromatogram of the mixed standard of 10 mg Kg^{-1} of FFA and HMF at 217 nm and 284 nm.

B. Figure of merit to chromatographic methods

Selectivity

Good analyte selectivity in the chromatographic system is evidenced by minimal or no variation in the retention time of the target analytes. Conversely, a method with reduced column stabilization times can lead to changes in time and area, causing alterations in pressure, mobile phase composition, and premature elution due to inadequate cleanliness, low ionic strength, or inadequate gradients. These conditions can lead to inaccurate and imprecise results, attributed to early or late elution of compounds from the matrix, co-elution of peaks, and changes in chromatographic peak areas. In this sense, the selectivity of the methods was checked according to the system's reproducibility about retention times and reproducibility between areas. On the other hand, the retention times ranged from 0.0%- 0.01% between the standard and samples, indicative of the stabilization of the chromatographic system under the conditions established in the determination section (pump, degasser, oven, and column).

The reproducibility of the chromatographic system was satisfactory; the variance between the areas of the chromatographic peaks showed a variation of less than 2%. This result indicates a good performance of all the components of the system (pump, degasser, column, injector, detector) and confirmation of the homogeneity of the extract and standard, which guaranteed consistent and reliable results. The theoretical plates indicated an excellent degree of specific sites (>10000), which gave rise to separation within the column (table 1), evidenced by good separation and retention of NFCs by both methods, increasing the reliability in the correct separation of compounds in the extracts.

Table 1. Suitability of the chromatographic system before starting the validation tests.

Parameter	Acrylamide	FFA	HMF
Theoretical plates (N)	120830	12468	10584
Asymmetry factor	1.30	1.02	1.02
Retention time (min)	1.55	4.99	3.66
System Reproducibility (%RSD)	0.02%	0.03%	0.47%

On the other hand, small changes introduced in the mobile phase can affect the quantification of NFCs derived from the co-elution of analytes in the same peak, changes in peak shape, and, consequently, in the areas under the curve. The asymmetry factor is a parameter that relates to the reproducibility of the results, obtaining less uncertainty when the peak is sharp because the Gaussian shape and widening influence the area under the curve. In this sense, each analyte obtained an asymmetry factor between 1.3 and 1.02 (table 1), evidencing an adequate symmetry [20] for acrylamide, FFA, and HMF, reducing the error in quantification and confirming its reproducibility. In our review, no articles were found that have published the suitability of the chromatographic system (theoretical plates and the asymmetry factor) for the determinations of these analytes in coffee and cocoa.

Linearity

A linear fit implies a proportional relationship between analyte concentration and instrumental response. The estimated pure error between the model and the replicates of the different concentration levels was acceptable within the normal distribution ($p<0.05$). This allows estimating the concentration of the NFCs with a high degree of confidence or probability that the assessed value is the calculated one. Additionally, the t-distribution test indicated that the instrumental response changes concerning the analyte concentration and is not

constant, with a significant slope for all predictive models (table 1).

Table 2. Evaluate linearity, LOD, and LOQ for quantifying acrylamide, HMF, FFA, and FA in cocoa and coffee.

Parameter	Acrylamide	FFA	HMF
β	4.04*[a]	107.38*(Kg mg^{-1}muA^{-1})	129.01*(Kg mg^{-1}muA^{-1})
α	0.03[n.s,b]	16.27[n.s.] (muA)	-6.68[n.s.] (muA)
Linearity	0.03-1.33[c]	10-30 mg Kg^{-1}	10-30 mg Kg^{-1}
R^2	0.9978	1.0000	0.9998
LOD (µg mL^{-1})	0.003	0.081	0.249
LOQ (µg mL^{-1})	0.011	0.272	0.832

ns: Insignificant ($p<0.05$)
* Significant ($p>0.05$)
[a]: The ratio of the peak areas of standard acrylamide to acrylamide-d3.
[b]: Adimensional.
[c]: The ratio between the concentration of standard acrylamide and acrylamide-d3.

On the other hand, the intercept for all analytes was insignificant in the model, indicating that it is very close to zero or is zero and can be neglected without affecting the estimated concentration. In addition, the coefficient of determination showed that the variance in the instrumental response can be explained by a factor greater than 0.99 concerning the variance of the FFA and HMF concentration and the relationship between acrylamide concentration and acrylamide d3. The residual plot allows us to identify that the data tends to take the values of the model when approaching zero, indicating that the obtained models are reliable for linear prediction [19].

LOD and LOQ

The LOD and LOQ were essential to know the limits for reliable quantification and proper use of analytes across methods. When this value approaches 0, it facilitates the determination and identification of trace amounts of analytes at lower concentrations, widening the field of action of the methods, not only for characterization but also for quality control in acceptance and rejection. This is implicitly related to the sensitivity of the selected technique. Mass spectrometry is more sensitive because the fragmentation pattern of a molecule is attributed to the types of bonds and spatial location of the bonds. This allows a higher precision in confirming the analyte's identity than DAD (Table 3). According to estimates, the equipment can detect acrylamide from 3 µg L^{-1} and 81 µg L^{-1} for FFA and 249 µg L^{-1} for HMF. In this sense, the sensitivity of the equipment was adequate because the methods allowed the identification of the compounds in both matrices according to the reported ranges of 222 to 922 µg kg^{-1} acrylamide and 1.2 to 7.3 nmol g^{-1} HMF cocoa beans [26] and 400 µg kg^{-1} of acrylamide and 418 µg g^{-1} FFA in coffee beans [8], [27].

Accuracy

Recovery was between 80% and 120%, within AOAC, 2015 recommended limits. This is comparable to 73.4 - 92.8 % and 97 % acrylamide in coffee and alkaline cacao beans [28]. 89.94-97.54% HMF and 99.34-100.47% HMF and FFA were found by [8] in roasted cocoa and coffee, respectively. The t Student tests confirmed that concentration did not affect the recoveries obtained [20]. In this sense, the methods are accurate (table 3).

Precision and intermediate precision

Precision and intermediate precision are adequate because they meet FDA recommendations [20], as the precision is lower than 16%RSD for acrylamide and 5%RSD for FFA and HMF. In addition, the intermediate precision for all analytes is less than 16%. This is similar to that reported by other authors in ranges of 0.6-14.23% RSD [8], [14], [29]. The mixture of cocoa genotypes could influence the high variability in accuracy because the composition changes between genotypes, generating an effect on the formation of NFCs. Nevertheless, the methodology used is sufficient to obtain an acceptable intermediate precision.

Table 3. Figure of merit of the methods for the determination of acrylamide, FFA, and HMF.

Food	Analyte	Recovery (%)	Precision (%RSD)	Intermedia precision (%RSD)	HorRat (%)
Coffee	Acrylamide	87.46-111.5	8.26	13.51	0.84
	FFA	80.9-101.8	1.97	2.93	0.98
	HMF	86.4-100.8	1.09	6.16	0.36
Cocoa	Acrylamide	86.9-90.7	8.91	15.80	0.98
	FFA	80.9-101.8	2.73	15.73	1.97
	HMF	91.7-100.9	2.85	13.43	2.00

Reproducibility

The HorRat value was equal to or less than 2 (table 3), indicating that the methods are potentially reproducible between laboratories. However, the HMF and FFA in cocoa are close to the upper limit, which can be reduced by modifying the technique, such as increasing the homogenization time of the raw material to decrease the variation [18], [19].

V CONCLUSIONS

Two methods for quantifying and detecting NFCs in coffee and cocoa were validated regarding selectivity, linearity, LOD, LOQ, accuracy, precision, and intermediate precision. Adding clean-up steps and optimizing the chromatographic system prevents misidentification and quantification. The methods analyzed were robust, able to withstand minor changes and remain accurate and reliable.

The LOQ and LOD obtained showed that the method can accurately detect and quantify relatively low concentrations of the analytes. Furthermore, it was demonstrated that consistent

and reliable results can be obtained with this methodology. Finally, developing a methodology that allows simultaneous identification to reduce analysis costs may be challenging. The mixture of extracts can be an alternative for the joint detection of NFCs because some reagents, such as PSA, can retain most of the HMF, reducing its recovery. However, ion exchange cartridges and activated carbon may be an option for interference cleaning and analyte concentration.

Finally, the validated methodologies may be applied in quality control, research and development laboratories belonging to the coffee and cocoa industry, subject to internal verification. This will facilitate the concentration of NFCs to be controlled, optimised roasting operating conditions (time, temperature, pressure) to be reduced, and NFCs to be ensured to be of the highest quality.

VI Acknowledgment

Sistema General de Regalías (SGR), Colombia, supported 431 work as part of the BPIN project 2020000100438.

References

[1] Y. F. Barrios-Rodríguez, N. Gutiérrez-Guzmán, F. Pedreschi, and M. S. Mariotti-Celis, "Rational design of technologies for the mitigation of neo-formed contaminants in roasted coffee," Trends Food Sci Technol, vol. 120, pp. 223–235, Feb. 2022, doi: 10.1016/j.tifs.2021.12.034.

[2] EFSA, "Scientific Opinion on acrylamide in food," EFSA Journal, vol. 13, no. 6, Jun. 2015, doi: 10.2903/j.efsa.2015.4104-9.

[3] J. Klaunig and J. Rice, "Genotoxicity and carcinogenicity of acrylamide," Toxicol Lett, vol. 189, p. S41, Sep. 2009, doi: 10.1016/j.toxlet.2009.06.130.

[4] H. Glatt, H. Schneider, M. Murkovic, B. H. Monien, and W. Meinl, "Hydroxymethyl-substituted furans: Mutagenicity in Salmonella typhimurium strains engineered for expression of various human and rodent sulphotransferases," Mutagenesis, vol. 27, no. 1, pp. 41–48, Jan. 2012, doi: 10.1093/mutage/ger054.

[5] T. Delatour, A. Desmarchelier, and R. H. Stadler, "Challenges in the measurement of acrylamide in food by confirmatory methods," Curr Opin Food Sci, vol. 48, p. 100951, Dec. 2022, doi: 10.1016/j.cofs.2022.100951.

[6] A. Desmarchelier et al., "Towards a consensus LC-MS/MS method for the determination of acrylamide in food that prevents overestimation due to interferences," Food Additives & Contaminants: Part A, vol. 39, no. 4, pp. 653–665, Apr. 2022, doi: 10.1080/19440049.2021.2022773.

[7] Q. Liu, P. Zhou, P. Luo, and P. Wu, "Occurrence of Furfural and Its Derivatives in Coffee Products in China and Estimation of Dietary Intake," Foods, vol. 12, no. 1, p. 200, Jan. 2023, doi: 10.3390/foods12010200.

[8] A. Albouchi and M. Murkovic, "LC method for the direct and simultaneous determination of four major furan derivatives in coffee grounds and brews," J Sep Sci, vol. 42, no. 9, pp. 1695–1701, May 2019, doi: 10.1002/jssc.201900061.

[9] Y. Huang et al., "Simultaneous Determination of Acrylamide and 5-Hydroxymethylfurfural in Heat-Processed Foods Employing Enhanced Matrix Removal—Lipid as a New Dispersive Solid-Phase Extraction Sorbent Followed by Liquid Chromatography–Tandem Mass Spectrometry," J Agric Food Chem, vol. 67, no. 17, pp. 5017–5025, May 2019, doi: 10.1021/acs.jafc.8b05703.

[10] A. Jozinović et al., "Simultaneous determination of acrylamide and hydroxymethylfurfural in extruded products by LC-MS/MS method," Molecules, vol. 24, no. 10, 2019, doi: 10.3390/molecules24101971.

[11] D. Sonawane, A. K. Sahu, T. Jadav, R. K. Tekade, and P. Sengupta, "Innovation in Strategies for Sensitivity Improvement of Chromatography and Mass Spectrometry Based Analytical Techniques," Crit Rev Anal Chem, vol. 53, no. 3, pp. 655–671, Apr. 2023, doi: 10.1080/10408347.2021.1969887.

[12] G. Wei, M. Dan, G. Zhao, and D. Wang, "Recent advances in chromatography-mass spectrometry and electronic nose technology in food flavor analysis and detection," Food Chem, vol. 405, p. 134814, Mar. 2023, doi: 10.1016/j.foodchem.2022.134814.

[13] A. Desmarchelier, J. Hamel, and T. Delatour, "Sources of overestimation in the analysis of acrylamide-in coffee by liquid chromatography mass spectrometry," J Chromatogr A, vol. 1610, p. 460566, Jan. 2020, doi: 10.1016/j.chroma.2019.460566.

[14] P. C. Aguas, M. J. Fitzhenry, G. Giannikopoulos, and P. Varelis, "Analysis of acrylamide in coffee and cocoa by isotope dilution liquid chromatography-tandem mass spectrometry," Anal Bioanal Chem, vol. 385, no. 8, pp. 1526–1531, Aug. 2006, doi: 10.1007/s00216-006-0560-9.

[15] A. D. Troise and A. Scaloni, "Quantitation of acrylamide in foods by high-resolution mass spectrometry," in Acrylamide in Food, Elsevier, 2024, pp. 565–579. doi: 10.1016/B978-0-323-99119-3.00014-X.

[16] A. Bebius et al., "Determination of Acrylamide in Coffee, Cereals, Baby Food, Cocoa, Dry Petfood, Potato Products, Vegetable Crisps, Biscuit, Tea, Nuts and Spices by LC-MS/MS in a Single Laboratory Validation: First Action 2023.01," J AOAC Int, Jan. 2024, doi: 10.1093/jaoacint/qsae007.

[17] M. Dziadosz, "Multiple analyte adduct formation in liquid chromatography-tandem mass spectrometry - Advantages and limitations in the analysis of biologically-related samples," Journal of Chromatography B, vol. 1084, pp. 1–3, May 2018, doi: 10.1016/j.jchromb.2018.03.027.

[18] AOAC International, "Appendix F: Guidelines for Standard Method Performance Requirements," in Official Methods of Analysis of AOAC International , 20th ed., Jr. Dr. George W. Latimer, Ed., 2016.

[19] B. Magnusson and U. Örnemark, The fitness for purpose of analytical methods : a laboratory guide to method validation and related topics. 2014.

[20] FDA, "FOOD AND DRUG ADMINISTRATION OFFICE OF REGULATORY AFFAIRS ORA Laboratory Manual Volume II Methods, Method Verification and Validation Sections in This Document," 2020.

[21] G. Sun, P. Wang, W. Chen, X. Hu, F. Chen, and Y. Zhu, "Simultaneous quantitation of acrylamide, 5-hydroxymethylfurfural, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine using UPLC-MS/MS," Food Chem, vol. 375, p. 131726, May 2022, doi: 10.1016/j.foodchem.2021.131726.

[22] F. Pedreschi, S. Mariotti, K. Granby, and J. Risum, "Acrylamide reduction in potato chips by using commercial asparaginase in combination with conventional blanching," LWT - Food Science and Technology, vol. 44, no. 6, pp. 1473–1476, Jul. 2011, doi: 10.1016/j.lwt.2011.02.004.

[23] N. J. Nielsen, K. Granby, R. V. Hedegaard, and L. H. Skibsted, "A liquid chromatography – tandem mass spectrometry method for simultaneous analysis of acrylamide and the precursors, asparagine and reducing sugars in bread," Anal Chim Acta, vol. 557, no. 1–2, pp. 211–220, Jan. 2006, doi: 10.1016/j.aca.2005.09.077.

[24] G. Strocchi, P. Rubiolo, C. Cordero, C. Bicchi, and E. Liberto, "Acrylamide in coffee: What is known and what still needs to be explored. A review," Food Chem, vol. 393, p. 133406, Nov. 2022, doi: 10.1016/j.foodchem.2022.133406.

[25] J. Wang et al., "A novel single step solid-phase extraction combined with bromine derivatization method for rapid determination of acrylamide in coffee and its products by stable isotope dilution ultra-performance liquid chromatography tandem triple quadrupole electrospray ionization mass spectrometry," Food Chem, vol. 388, p. 132977, Sep. 2022, doi: 10.1016/j.foodchem.2022.132977.

[26] R. F. Peña-Correa, B. Ataç Mogol, and V. Fogliano, "The impact of roasting on cocoa quality parameters," Crit Rev Food Sci Nutr, pp. 1–14, Nov. 2022, doi: 10.1080/10408398.2022.2141191.

[27] H. Endeshaw and A. Belay, “Optimization of the roasting conditions to lower acrylamide content and improve the nutrient composition and antioxidant properties of Coffea arabica,” PLoS One, vol. 15, no. 8, p. e0237265, Aug. 2020, doi: 10.1371/journal.pone.0237265.
[28] Z. Li, C. Zhao, and C. Cao, “Production and Inhibition of Acrylamide during Coffee Processing: A Literature Review,” Molecules, vol. 28, no. 8, p. 3476, Apr. 2023, doi: 10.3390/molecules28083476.
[29] S. Park, A. Jo, and K.-G. Lee, “Effect of various roasting, extraction and drinking conditions on furan and 5-hydroxymethylfurfural levels in coffee,” Food Chem, vol. 358, p. 129806, Oct. 2021, doi: 10.1016/j.foodchem.2021.129806.