

Article

DETERMINATION OF SWEETENERS IN WINE BY LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY (LC/MS)

DETERMINAÇÃO DE EDULCORANTES EM VINHO POR CROMATOGRAFIA LÍQUIDA ACOPLADA A ESPECTROMETRIA DE MASSA (LC/MS)

Nuno Neves^{1,*}, Natália Ribeiro², Cristina Esteves², Paulo Barros²

¹ Sogrape Vinhos S.A., Rua 5 de Outubro, 4527, 4430-809 Avintes, Vila Nova de Gaia, Portugal.

² Instituto dos Vinhos do Douro e do Porto, I.P., Rua Ferreira Borges, 27, 4050 Porto, Portugal.

* Corresponding author: Tel.: + 351 962 373 704; e-mail: nuno.neves@sogrape.pt

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SUMMARY

Sweeteners are food additive substances that give a sweet taste to foods but their use in oenological practices is forbidden. Making use of the capabilities of liquid chromatography coupled with mass spectrometry, a method for wine analysis was developed and validated for the detection and quantitation of some of the most widely used sweeteners: aspartame, potassium acesulfame, sodium cyclamate, saccharin, sucralose and stevioside. A matrix-matched calibration was used for all compounds obtaining a linear concentration range from 50 µg/L to 1000 µg/L. The limit of detection ranged from 0.002 mg/L to 0.014 mg/L, and the limit of quantification varied between 0.005 mg/L and 0.048 mg/L. Precision and recovery were assessed for 50 µg/L, 250 µg/L and 1000 µg/L with repeatability and intermediate precision values from 0.6% to 21.6% and 2.7% to 26.4% respectively, and recoveries ranging from 60% to 126%. These results were achieved using minimal sample preparation with a fast and high throughput method that is applicable to a wide range of wine matrices.

RESUMO

Os edulcorantes são aditivos alimentares que conferem sabor doce aos alimentos, mas a sua utilização em práticas enológicas é proibida. Utilizando as capacidades da cromatografia líquida acoplada à espectrometria de massa, foi desenvolvido e validado um método de análise de vinhos para a deteção e quantificação de alguns dos edulcorantes mais utilizados: aspartame, acesulfame de potássio, ciclamato de sódio, sacarina, sucralose e stevioside. Foi utilizada uma calibração em matriz para todos os compostos numa gama linear de concentrações entre 50 µg/L e 1000 µg/L. O limite de deteção variou entre 0,002 mg/L e 0,014 mg/L, e o limite de quantificação variou entre 0,005 mg/L e 0,048 mg/L. A precisão e a recuperação foram determinadas para as concentrações de 50 µg/L, 250 µg/L e 1000 µg/L, com repetibilidade e precisão intermédia compreendidas entre 0,6% e 21,6% e 2,7% e 26,4% respetivamente, e recuperações entre 60% e 126%. Estes resultados foram obtidos recorrendo a uma preparação de amostra simples num método rápido e de elevada eficiência de análise, sendo aplicável a uma vasta gama de vinhos.

Keywords: sweeteners, wine, method validation, liquid chromatography, mass spectrometry.

Palavras-chave: edulcorantes, vinho, validação de método, cromatografia líquida, espectrometria de massa.

INTRODUCTION

Sweeteners are food additive substances that are used to impart a sweet taste to foods or balance the taste properties of the product (Regulation (EC) No 1333/2008, 2008; OIV, 2021). Different classifications are applied depending on the sweetening strength of a particular sweetener. With the sweetness potency of sucrose considered as a reference, sweeteners with equivalent or lower sweetening strength are bulk sweeteners (Shah and Jager, 2017). These are used as food preservatives

or to provide texture, mouthfeel and volume to food (Saltmarsh and Insall, 2013). In contrast, intense sweeteners have sweetness strength much higher than sucrose. Due to the characteristic intense sweet taste, they are used as replacements for sugars in the production of foods (Regulation (EC) No 1333/2008, 2008; Shah and Jager, 2017). These food additives can be of natural, synthetic or semi-synthetic origin and are highly appealing to the trending low calorie products (DuBois and Prakash, 2012; Sylvetsky and Rother, 2016; Shah and Jager, 2017). High intensity sweeteners are non-nutritive

or low caloric compounds and, even though they are much more potent than natural carbohydrates and therefore used in small amounts, they often produce an undesirable aftertaste (Kubrica *et al.*, 2015). To limit this outcome and to benefit from synergistic effects, sweeteners are often used in combination (Shah and Jager, 2017). Among the most used high intensity sweeteners one can count aspartame, potassium acesulfame, sodium cyclamate, saccharin, sucralose and stevia glycosides such as stevioside (Shankar *et al.*, 2013; Mbambo *et al.*, 2020). Stevioside is of natural origin while the remaining are artificial sweeteners.

The sweetening strength of each sweetener is characterized by an index of relative sweetness. Table I shows the sweetening strengths of the abovementioned sweeteners, taking sucrose sweetness as a reference value of 1. Thus, the higher the value, the more potent the sweetener is and the smaller the amount needed to produce the desired sweet taste. This implies that sweeteners need to be analysed in much lower concentrations when compared to sugars, which also creates additional analytical challenges.

Table I
Sweeteners relative strength

Sweetener	Relative sweetness (sucrose = 1)
Acesulfame K	200
Aspartame	200
Cyclamate Na	30
Saccharin	200-700
Sucralose	600
Stevioside	200-400

Source: U.S. FDA, 2018; Rocha *et al.*, 2005.

In the past, the most used method for the analysis of sweeteners was thin-layer chromatography (TLC) but several other methods were published, including a variety of analytical techniques such as capillary electrophoresis (CE), gas chromatography (GC), ion chromatography (IC), ion-pair chromatography and hydrophilic interaction liquid chromatography (HILIC) (Shah and Jager, 2017; Li *et al.*, 2020). Nowadays, the most used method is reverse-phase high performance liquid chromatography (RP-HPLC) combined with different choices of detectors (Kubrica *et al.*, 2015). Several authors reported the use of ultra-violet-visible detectors (UV-vis), diode array detector (DAD), electrochemical detector (ECD), evaporative light-scattering detection (ELSD), charged aerosol detector (CAD) and mass spectrometry (MS) (Zou *et al.*, 2018; Li *et al.*, 2020). In the analysis of sweeteners there are many challenges to be faced and every method or technique has its own limitations. It is highly desirable for a method to be able to perform simultaneous analysis on several sweeteners. However, due to the observed variability of physicochemical, electrochemical and spectral

properties, the majority of the published methods addresses only one particular sweetener or a simple mixture (Li *et al.*, 2020). For chromatographic methods, it is usually necessary to have baseline separation of the different compounds which is difficult to achieve. In some detectors, like UV and DAD, the poor or lack of response of some compounds may require derivatization and, particularly for the former, there may be issues with specificity (Shah and Jager, 2017). Other challenges may be considered such as the low volatility of the sweeteners when the use of GC is intended, poor selectivity for ELSD, hard to achieve robustness for CE or poor detection limits for CAD (when compared to MS) (Shah and Jager, 2017). Today, the method of choice for quantitation and confirmation of sweeteners in foods is the RP-HPLC coupled with MS or MS/MS (tandem MS) and usually with electrospray ionization (ESI) (Kubrica *et al.*, 2015; Shah and Jager, 2017; Li *et al.*, 2020). This technique allows the direct and simultaneous analysis of multiple compounds and provides high specificity, high sensitivity and robustness (Shah and Jager, 2017). Nevertheless, MS has its own limitations and often one has to take into account ion suppression phenomena. One of the biggest challenges in the analyses of food products is overcoming the matrix effect (Shah and Jager, 2017). Matrix effect can affect the analytical calibration procedures and influence the results during method validation. To deal with the matrix, several authors resort to sample preparation steps such as liquid-liquid extraction, solid-liquid extraction, solid phase extraction (SPE), dispersive SPE or even dilution with water or mobile phase and filtration (Shah and Jager, 2017; Li *et al.*, 2020). Notwithstanding its importance to the analytical method, in many regulatory documents, matrix effect is not addressed in sufficient detail. In fact, many of them only address matrix effect superficially and in others the information is missing. Additionally, there is no consensus among regulatory documents regarding the evaluation and management of matrix effects (Raposo and Barceló, 2021). Among the method validation guidelines, SANTE can be considered as one of the most comprehensive regulatory documents concerning matrix effects (Raposo and Barceló, 2021).

At the international level, the International Organisation of Vine and Wine (OIV) establishes provisions regarding the definition of vitivincultural products and oenological practices and treatments, accepted or otherwise. As stated in the "International Code of Oenological Practices" (OIV, 2021), the addition of high intensity sweeteners is foreseen only for aromatized wines, beverages based on vitivincultural products and wine-based beverages. Moreover, even if the former condition is met, the sweeteners must also comply with the regulations of producing and consumer countries. In wine, products other than grape must,

concentrated grape must and rectified concentrated grape must are forbidden to be used in sweetening oenological practices (Commission Delegated Regulation (EU) 2019/934, 2019). Therefore, it is evident that the use of sweeteners, and in particular high intensity sweeteners, is strictly forbidden for this commodity. Despite the use of sweeteners in wine being totally prohibited, they are often used to produce counterfeit wines (Geana *et al.*, 2012). Besides Geana *et al.* (2012), Zou *et al.* (2018) reported one out of 10 wines detected with sodium cyclamate (0.28 mg/L) and neotane (0.36 mg/L), and Li *et al.* (2020) reported five wines detected with sweeteners. Unlike the beverages in which sweetener addition is permitted and regulated, bearing in mind that wines may not contain any sweeteners, even at trace concentrations, a method to detect and quantify these compounds at extremely lower levels is necessary.

Concerning the analysis of sweeteners in wines, the literature is scarce, especially when considering simultaneous analysis of natural (e.g., stevia-based sweeteners) and synthetic high intensity sweeteners (Kubrica *et al.*, 2015; Zou *et al.*, 2018). Kubrica *et al.* (2015) presented an HPLC method coupled with ESI and a triple quadrupole MS instrument for the analysis of 14 sweeteners. The method focused on the application of the same method for both synthetic sweeteners and steviol glycosides (natural sweeteners). However, the reported limit of quantitation range was very high (in the mg/L range) because the samples were diluted 100-fold to avoid matrix effects. Also, the validation was made with a soft drink. Zou *et al.* (2018) used the same type of instrument to analyse eight sweeteners with very good results (limit of detection varying from 0.002 mg/L to 0.0035 mg/L) but validation was performed in beer and the study of the applicability of the method to wine is limited. Using a different approach, Li *et al.* (2020) successfully employed a direct analysis in real time MS (DART-MS) instrument. The method allowed for LOQ between 0.002 mg/L and 0.2 mg/L.

The aim of this work was to develop a method directed to wine analysis that would enable the detection of unauthorized oenological practices of sweetener addition to wine. This method should have good performance in the evaluation of wines with a wide variability of matrix characteristics. Additionally, the method should be thought out in terms of its applicability in routine analysis for wine control. Therefore, cumbersome sample treatment should be avoided and a fast method should be favoured. Nevertheless, one should not compromise low limit of detection and set the target well below the sensory threshold of the considered sweeteners as referred to in the literature (Nelson, 2000; Geana *et al.*, 2012; Shankar *et al.*, 2013; Carochi *et al.*, 2017; Shah and Jager, 2017; U.S. FDA, 2018). For that purpose, the authors developed a fast analytical method with minimal sample preparation and

without employing expensive labelled internal standards that allows simultaneous detection and quantitation of all the above-mentioned compounds at very low levels using liquid chromatography coupled with mass spectrometry (LC/MS). In liquid chromatography (LC), separation was performed using a reverse phase (RP) column and detection was accomplished by mass spectrometry (MS) according to the compounds' mass to charge ratio (m/z). The MS data combined with the retention time (RT) were used for the identification and quantitation of sweeteners.

The method herein presented is currently incurring in the step process of the OIV in order to be approved as a Resolution with the ensuing inclusion in the "Compendium of International Methods of Analysis of Wines and Musts".

MATERIALS AND METHODS

Standards for the artificial sweeteners aspartame, potassium acesulfame, sodium cyclamate, saccharin and sucralose and for the natural sweetener stevioside, all of analytical grade, were purchased from Sigma Aldrich[®]. Acetonitrile of LC/MS grade was purchased from Honeywell Fluka[™], and formic acid was purchased from Merck[®]. Water was purified with a Milli-Q advantage A10 system from Millipore[®]. Samples for wine analysis were obtained from commercially available finished bottled wines. Wines were verified for the presence of sweeteners beforehand.

Sample preparation

Each wine sample was prepared by filtration with a 0.2 μm polypropylene membrane syringe filter prior to injection. When necessary, samples were degassed beforehand in an ultrasound bath.

MS parameters

For the detection and quantitation of sweeteners, it was used a combined quadrupole and time-of-flight (QTOF) instrument, maXis impact[™], from Bruker. The instrument was operated in ESI negative mode, with a source temperature of 200 °C and a capillary voltage of 3000 V. Full scan and MS/MS acquisition modes were used with a spectra rate of 2 Hz and collision energy of 30 eV for fragmentation. Further calculations were based on the full scan signal while the MS/MS data was used for peak confirmation.

LC parameters

Separation was performed on a Dionex UltiMate[™] 3000 UHPLC system from Thermo Scientific[™]. A reverse phase C8 2.1 mm x 100 mm, 1.9 μm column (from Thermo Scientific[™]) was used to separate all the compounds using mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) with an initial flow rate of 0.4 mL/min of 10% B and raising %B up to 40% in 3

min while maintaining flow rate. Then 99% B was held for 1 min at 0.4 mL/min and for the subsequent 1.5 min the flow raised to 0.8 mL/min. At this point the column was re-equilibrated at 10% B with 0.5 mL/min for 4 min and back to the initial conditions for an additional 0.5 min.

The column temperature was held at 30 °C and the injection volume used was 2 µL.

Standard solutions

Individual standard solutions at 1 g/L were prepared by dissolving 10.0 mg of each sweetener in 10 mL volumetric flasks and filling up to the mark with water. Wine samples free of sweeteners were used as calibration matrix; thus, the calibration standards were prepared in wine by diluting the appropriate amount of standard solution to obtain the concentrations 50 µg/L, 100 µg/L, 500 µg/L and 1000 µg/L of each sweetener. Standards and calibration solutions were kept in the fridge at approximately 6 °C. Aspartame solutions are unstable in acid media; therefore, they must be prepared fresh each time the standard is analysed.

Calibration standards were prepared and analysed by LC/MS as any other sample. However, while samples were injected twice, calibrations were made by triplicate injections and considering all the points for the calibration curve.

RESULTS AND DISCUSSION

The aim of this method is to search and quantify compounds that would not be present in wine if oenological practices are followed according to the law in force. However, one should not assume the absence of these compounds. Therefore, wines were tested for potential interferences in the identification and measurement of sweeteners. The identification characteristics were obtained from spiking of wine samples with a standard for each sweetener. The data gathered was the RT and the m/z of the precursor ion. The confirmation of the peak identity was achieved by analysing product ions (MS/MS data) of each sweetener eluted at their exact RT. Figure 1 shows the chromatograms for the identification of each sweetener, with the superposition of the precursor and product ions. The chromatograms were built out of the acquisition spectra from mass spectrometry and the compounds displayed at their specified m/z values with a tolerance of ± 3 mDa.

For aspartame, saccharin, sucralose and stevioside, the measured ion was the monoisotopic ion in the form [M-H]⁻ and in the form [M]⁻ for acesulfame K and cyclamate Na. The highest signal between precursor and product ions was selected as the quantitative ion. Table II compiles the identification information.

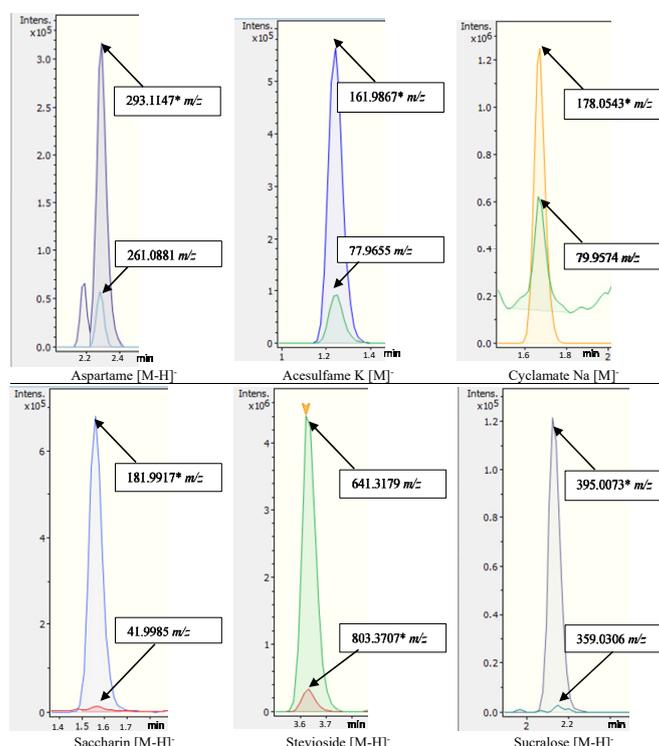


Figure 1. Precursor and product ions for peak identification and confirmation. *Precursor ions.

The system was tested by injection of blank samples (purified water) to check for interferences in the

identification and quantitation. Also, the water blank was compared against a wine sample without

sweeteners and the same sample spiked with 250 µg/L of each sweetener. In Figure 2 the signals are shown for the m/z value of each sweetener and within their respective RT range (also with ± 3 mDa

tolerance). The chromatograms are stacked instead of overlaid to better distinguish the three signals, particularly at the baseline.

Table II

Data for the identification of sweeteners: RT, precursor ion m/z and product ion m/z¹

Sweetener	RT min	Ion	Precursor m/z	Product m/z
Acesulfame K	1.24	[M] ⁻	<u>161.9867</u>	77.9655
Aspartame	2.30	[M-H] ⁻	<u>293.1143</u>	261.0881
Cyclamate Na	1.66	[M] ⁻	<u>178.0543</u>	79.9574
Saccharin	1.55	[M-H] ⁻	<u>181.9917</u>	41.9985
Sucralose	2.14	[M-H] ⁻	<u>395.0073</u>	359.0306
Stevioside	3.63	[M-H] ⁻	803.3707	<u>641.3026</u>

¹ The ions selected for quantitation are underlined.

From Figure 2 it is evident the lack of signal from the water blank and from the sample. Additionally, there is a good isolation of the sweeteners' signal from the compounds of the matrix. All the wine samples were analysed and none of the sweeteners were detected in any of them. Therefore, in this study, all the samples for which the presence of sweeteners is reported refers to wine samples spiked with some concentration of sweetener. As for the set of samples of this study, they were selected with the

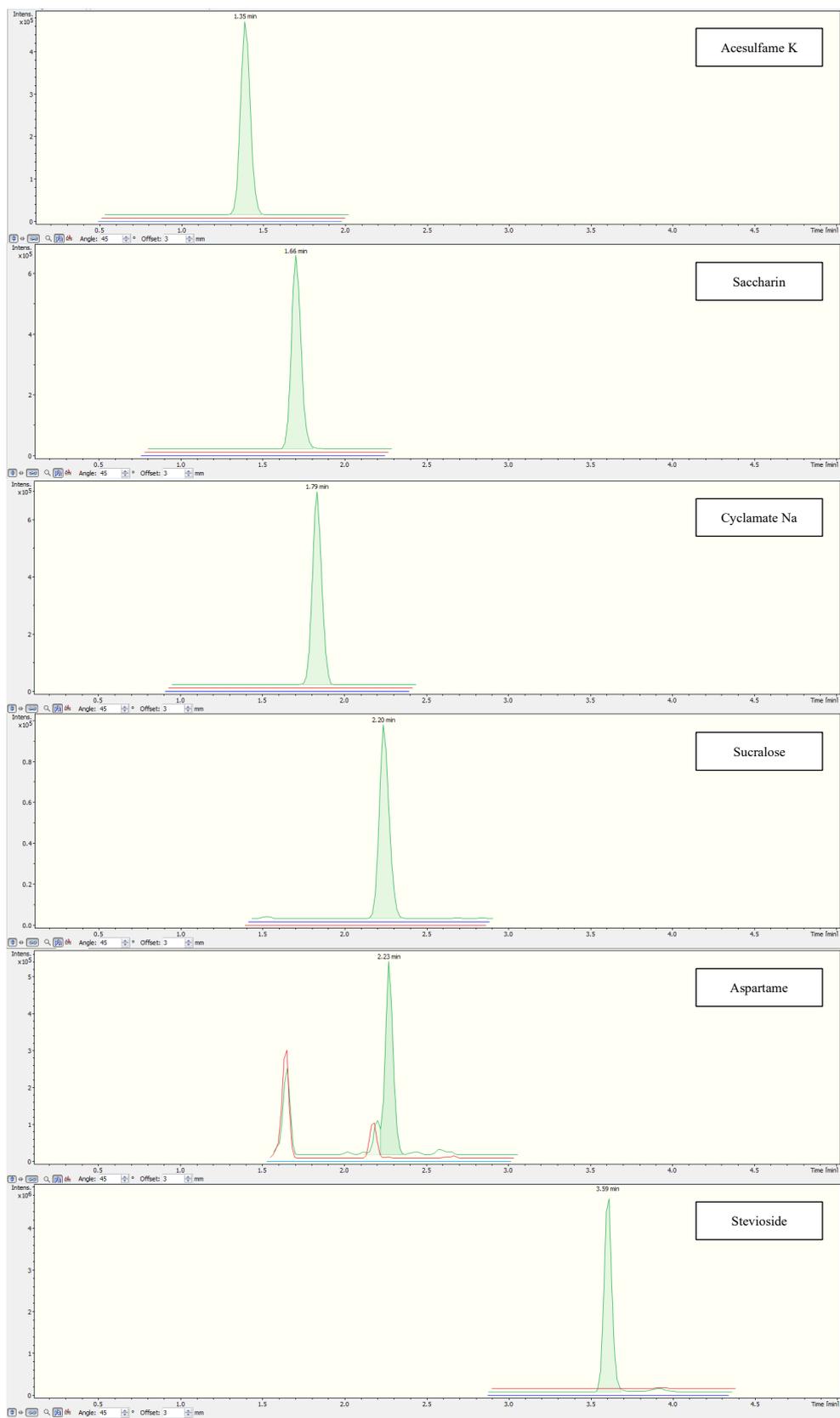
purpose of imposing a wide variability of characteristics to obtain a highly comprehensive method. In total, 43 different wines, 20 red wines, 10 rosé wines and 13 white wines, were gathered from several regions and had very distinct features. The wines used for precision assessment were different from those used for calibration while maintaining the abovementioned wide variability of characteristics (Table III).

Table III

Main characteristics of the matrices

	Red wine		Rosé wine		White wine	
	Calibration	Precision	Calibration	Precision	Calibration	Precision
Açores						1
Alentejo	2	2			1	1
Bairrada		1				
Dão	1	2				1
Number of wines per region						
Douro	1	3	1	2	1	
Lisboa		1				1
Valladolid		1				
Vinhos Verdes			1		1	3
Other ⁽¹⁾	2	4	2	4	1	2
Alcoholic strength by volume (% V/V)	12.9 - 14.3	12.1 - 17.2	9.8 - 11.6	10.3 - 12.6	8.7 - 12.7	8.7 - 13.6
Sugar content (g/L; glucose + fructose)	0.5 - 12.8	0.7 - 108	0.7 - 28.8	0.7 - 21.3	0.7 - 17.1	0.2 - 11.7
Total Acidity (g/L; tartaric acid)	4.8 - 5.5	4.6 - 6.4	5.5 - 6.0	4.7 - 6.0	5.2 - 5.7	5.6 - 7.1
pH	3.7 - 3.8	3.5 - 3.8	3.2 - 3.3	3.2 - 3.5	3.2 - 3.3	3.2 - 3.4
Colour Intensity (Abs 420 + 520 + 620 nm)	6.8 - 13.4	2.36 - 16.17	0.1 - 0.5	0.1 - 0.5	0.04 - 0.06 ⁽²⁾	0.03 - 0.29 ⁽²⁾

⁽¹⁾ Without geographical indication; ⁽²⁾ Absorbance at 420 nm instead of colour intensity.



Blue Water Blank **Red** Wine sample without sweeteners **Green** Wine sample spiked with 250 µg/L of each sweetener

Figure 2. Chromatogram stacked view showing the comparison of signals from the water blank, a wine sample and the same sample spiked with 250 µg/L of each sweetener.

Besides considering different wine types (red, rosé and white), among each category there is also great divergence which is illustrated by the range of values of each parameter. There are dry and sweet wines, very light and very intense coloured, high and low alcoholic content and also differences in acidity.

Internal validation

Linearity

Linearity was tested for two concentration ranges: one from 50 µg/L to 1000 µg/L, and another from 50 µg/L to 5000 µg/L. In the latter case, many of the compounds did not maintain linearity through the entire range of calibration, thus this range was rejected and the range from 50 µg/L to 1000 µg/L was established for all the compounds.

Calibration

A matrix-matched calibration approach was used to deal with matrix effects and the calibration concentrations were chosen based on the sweetening index of the studied sweeteners. The first points were closer together to better characterize the lower concentration range focusing the method on analysing trace amounts of sweeteners and the other points were more spaced to extend the calibration range. In addition, the use

of few calibration points makes it easier to apply the method in routine analysis. In line with the focus on low concentration, it was chosen not to apply sample dilution providing easier and fast sample preparation while at the same time guaranteeing low limits of detection and quantitation.

A total of 14 independent calibrations were made counting six red wines, four rosé wines and four white wines. Then, for each compound, calibrations were made considering three different approaches: i) one unified calibration for all the matrices; ii) two groups of matrices consisting in one group for white wines and another group with the remaining wines (red wines and rosé wines); iii) three groups of matrices consisting of white wines, rosé wines and red wines.

The results of the validation study that showed better performance are presented (ii and iii). According to the selected calibration conditions, for potassium acesulfame, saccharin and sucralose, calibration functions and subsequent calculations were performed considering one group for white wines and a second group with the remaining matrices, red wines and rosé wines. For aspartame, sodium cyclamate and stevioside three groups of matrices were considered: red wines, rosé wines and white wines (Table IV).

Table IV

Calibration scheme for each compound depending on matrix

Calibrations	Individual			Combined
	White wine	Rosé wine	Red wine	Red wines + Rosé wines
Acesulfame K	X			X
Aspartame	X	X	X	
Cyclamate Na	X	X	X	
Saccharin	X			X
Stevioside	X	X	X	
Sucralose	X			X

For each calibration group, homogeneity of variances was tested by comparison of the variance of the residuals at the first and last calibration levels. Also, normal distribution of the residuals was assessed using a Q-Q plot plotting the normal distribution theoretical quantiles against the standardized residuals. In all the cases the results showed that there is heteroskedasticity of the data and that the distribution of the residuals is close to

normality. As an example, sucralose for the group of red and rosé wines at a concentration range from 50 µg/L to 1000 µg/L is presented in Figure 3.

Given the heteroskedasticity and normal distribution of the residuals, the regression model employed was the weighted least square regression. Several weighting factors were considered: $1/x^{0.5}$, $1/x$, $1/x^2$, $1/y^{0.5}$, $1/y$ and $1/y^2$. A regression model was made

for each weighting factor and the sum of relative errors was assessed according to Equation 1.

$$\text{Relative error} = \sum(C_{\text{regression}} - C_{\text{nominal}})/C_{\text{nominal}} \quad \text{Eq. 1}$$

Where C_{nominal} is the concentration of the standard, and $C_{\text{regression}}$ is the measured standard concentration through the regression. The model that produced the lowest sum of relative errors was the one considered to give the best fit for the data and thus selected.

Results were calculated from the calibration curve which was obtained with the amount ($\mu\text{g/L}$) vs the peak area of each sweetener, and concentrations were expressed in $\mu\text{g/L}$ without decimals.

Limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) were obtained through calculation from the calibration curves (Table V).

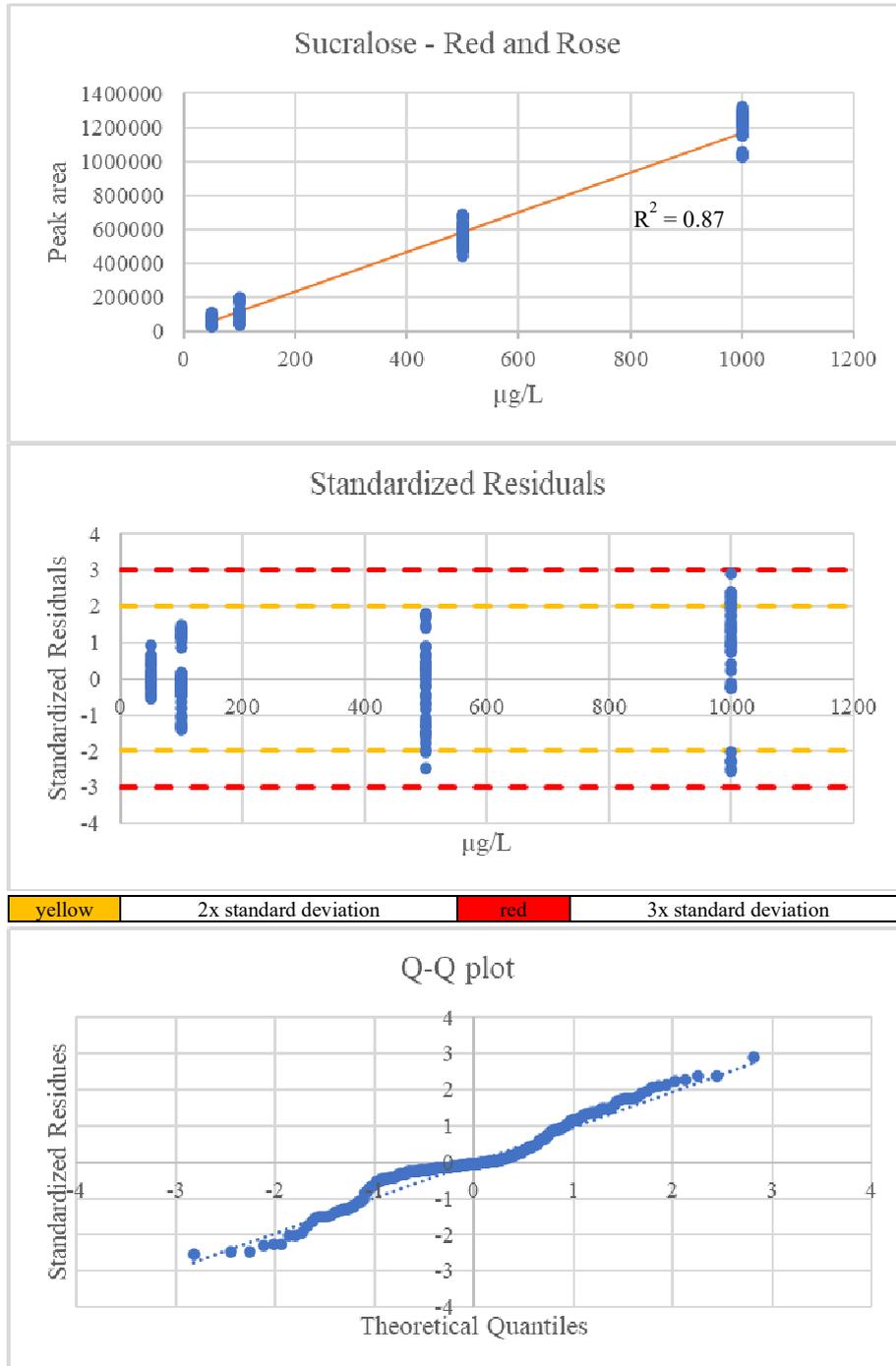


Figure 3. Calibration curve, standardized residuals and Q-Q plot for the combined red and rosé wines calibration for sucralose.

The obtained values of LOD varying from 0.002 mg/L to 0.014 mg/L allowed the detection of trace amounts of sweeteners and are in line with the lowest values found in the literature (Zou *et al.*, 2018; Li *et al.*, 2020).

Precision

Precision was assessed with wine samples spiked at three concentration levels: 50 µg/L, 250 µg/L and 1000 µg/L. The same spiking levels were used for both intra-day and inter-day precision. Intra-day precision (repeatability) was based on the measurements of eight replicates of wine samples under repeatability conditions while for inter-day precision (intermediate precision) 10-12 points were determined, each point on a different day.

The concentrations 50 µg/L and 1000 µg/L were chosen to represent the lowest and the highest point in the calibration range, respectively. The point 250 µg/L is in between.

For repeatability, the gathered data included the mean concentration measured in each spiked sample, the standard deviation, the relative standard deviation for repeatability (RSDr%) and the Horwitz Ratio for repeatability (HorRat(r)). Horwitz Ratio for repeatability is calculated from Equation 2 (Horwitz and Albert, 2006).

$$\text{HorRat}(r) = \text{RSDr}\% / \text{PRSD}\%_{\text{R}} \quad \text{Eq. 2}$$

Where RSDr% is the relative standard deviation for repeatability conditions and PRSD%_R is the predicted RSD% for reproducibility obtained from the Horwitz equation (Horwitz and Albert, 2006) (Equation 3).

$$\text{PRSD}\%_{\text{R}} = 2C^{-0.15} \quad \text{Eq. 3}$$

Where C is the concentration value expressed as a dimensionless mass fraction.

Table V

LOD and LOQ values obtained for each compound

	LOD (mg/L)			LOQ (mg/L)		
	White wine	Rosé wine	Red wine	White wine	Rosé wine	Red wine
Acesulfame K	0.003	0.003		0.011	0.011	
Aspartame	0.004	0.006	0.004	0.014	0.019	0.014
Cyclamate Na	0.002	0.005	0.004	0.006	0.015	0.014
Saccharin	0.002	0.005		0.006	0.016	
Stevioside	0.002	0.002	0.005	0.005	0.005	0.016
Sucralose	0.014	0.007		0.048	0.022	

The repeatability results are compiled in Table VI with the data sorted by wine type.

Each row of Table VI refers to a different spiked wine and the columns separate the sweetener compounds. The differences between replicates can be evaluated by analysing the residual standard deviation values for repeatability (RSDr%). From the results, as it is expected, the RSDr% values increased for lower concentrations. Repeatability values for acesulfame K, saccharin and cyclamate Na were under 5.0%. The results for stevioside were similar with the exception of one value that is slightly higher with a value of 11.8%. For sucralose and aspartame, the differences in the RSDr% values between the 50 µg/L concentration and the remaining levels are more evident. The obtained HorRat (r) results ranged from 0.03 to 0.87, less than 2, therefore within the performance criteria (Commission Regulation (EC) No 401/2006). Some studies, such as that by Zou *et al.* (2018), presented

slightly better RSDr% values but they were measured for concentrations higher than 200 µg/L and using diluted samples. On the other hand, the obtained RSDr% values are better than those of Li *et al.* (2020).

For intermediate precision, the same equation (Equation 2) is applicable by substituting the RSDr% for the RSD%. Table VII shows the results for intermediate precision sorted by wine type.

The recovery percentage, the relative standard deviation (RSD%) and the Horwitz Ratio (HorRat) were also calculated for each case.

The RSD% values for intermediate precision were, in general, slightly higher than the repeatability results. This behaviour is normal given the different conditions from day to day in which the method was tested. The values range from 2.7% to 26.4%, and the HorRat values varied from 0.11% to 1.26%. As observed for the repeatability, these values are also

Table VI

Repeatability values for all compounds at three spiking levels

Spiking	Sample	Acesulfame-K			Aspartame			Cyclamate-Na			Saccharin			Stevioside			Sucralose		
		Recovery	RSDr	HorRat (r)	Recovery	RSDr	HorRat (r)	Recovery	RSDr	HorRat (r)	Recovery	RSDr	HorRat (r)	Recovery	RSDr	HorRat (r)	Recovery	RSDr	HorRat (r)
50 µg/L	W1	89 %	3.2 %	0.13	68 %	21.6 %	0.87	103 %	1.9 %	0.08	89 %	3.3 %	0.13	83 %	1.0 %	0.04	106 %	10.0 %	0.40
	W2	84 %	5.0 %	0.20	101 %	8.3 %	0.33	100 %	2.9 %	0.12	91 %	3.0 %	0.12	86 %	1.0 %	0.04	103 %	15.1 %	0.61
	W3	98 %	1.6 %	0.06	91 %	14.7 %	0.59	101 %	2.9 %	0.11	119 %	2.4 %	0.10	60 %	2.2 %	0.09	105 %	15.4 %	0.62
250 µg/L	W4	93 %	1.4 %	0.06	95 %	11.6 %	0.46	104 %	1.1 %	0.04	86 %	2.4 %	0.09	105 %	0.8 %	0.03	88 %	4.9 %	0.20
	W5	83 %	2.5 %	0.10	92 %	3.3 %	0.13	99 %	1.7 %	0.07	86 %	2.4 %	0.10	106 %	11.8 %	0.47	90 %	12.2 %	0.49
	W6	96 %	1.1 %	0.04	94 %	4.6 %	0.19	99 %	1.4 %	0.06	101 %	1.5 %	0.06	81 %	0.9 %	0.04	89 %	2.9 %	0.12
1000 µg/L	W7	104 %	1.3 %	0.05	98 %	3.0 %	0.12	109 %	1.1 %	0.04	92 %	2.3 %	0.09	109 %	1.2 %	0.05	99 %	3.0 %	0.12
	W8	93 %	1.5 %	0.06	97 %	1.8 %	0.07	104 %	1.7 %	0.07	91 %	2.6 %	0.10	112 %	1.2 %	0.05	97 %	4.5 %	0.18
	W9	106 %	1.5 %	0.06	98 %	2.4 %	0.09	105 %	1.4 %	0.06	105 %	2.0 %	0.08	86 %	0.8 %	0.03	102 %	3.1 %	0.12
50 µg/L	Ro1	98 %	4.1 %	0.17	75 %	8.0 %	0.32	84 %	3.9 %	0.16	116 %	2.4 %	0.10	99 %	1.7 %	0.07	70 %	11.7 %	0.47
	Ro2	104 %	2.3 %	0.09	85 %	6.9 %	0.28	85 %	3.0 %	0.12	112 %	3.5 %	0.14	77 %	3.4 %	0.14	86 %	5.0 %	0.20
	Ro3	107 %	2.6 %	0.10	82 %	10.6 %	0.43	88 %	1.7 %	0.07	112 %	1.1 %	0.04	81 %	2.2 %	0.09	71 %	6.2 %	0.25
250 µg/L	Ro4	99 %	1.2 %	0.06	80 %	3.4 %	0.17	93 %	1.2 %	0.06	121 %	1.8 %	0.09	95 %	1.1 %	0.06	86 %	3.3 %	0.17
	Ro5	99 %	1.4 %	0.07	84 %	2.5 %	0.13	91 %	2.0 %	0.10	110 %	1.3 %	0.07	102 %	2.1 %	0.11	94 %	3.1 %	0.16
	Ro6	99 %	1.6 %	0.08	84 %	2.8 %	0.14	93 %	1.9 %	0.10	111 %	1.7 %	0.09	114 %	2.5 %	0.13	78 %	4.0 %	0.20
1000 µg/L	Ro7	106 %	1.3 %	0.08	83 %	2.5 %	0.16	98 %	1.5 %	0.10	126 %	2.3 %	0.14	93 %	1.1 %	0.07	94 %	2.3 %	0.14
	Ro8	109 %	1.2 %	0.08	90 %	3.8 %	0.24	99 %	0.6 %	0.04	119 %	2.1 %	0.13	110 %	0.9 %	0.06	108 %	2.6 %	0.16
	Ro9	110 %	1.4 %	0.09	92 %	2.5 %	0.15	100 %	1.3 %	0.08	120 %	2.1 %	0.13	111 %	1.6 %	0.10	91 %	2.1 %	0.13
50 µg/L	R1	112 %	2.1 %	0.08	92 %	18.5 %	0.74	103 %	2.4 %	0.10	94 %	3.0 %	0.12	120 %	1.5 %	0.06	100 %	15.3 %	0.61
	R2	101 %	3.9 %	0.16	103 %	6.3 %	0.25	110 %	2.4 %	0.10	88 %	1.1 %	0.04	80 %	1.8 %	0.07	92 %	6.9 %	0.28
	R3	115 %	2.4 %	0.10	100 %	16.2 %	0.65	108 %	2.6 %	0.10	92 %	3.2 %	0.13	86 %	1.0 %	0.04	96 %	14.1 %	0.57
250 µg/L	R4	110 %	1.2 %	0.06	91 %	7.4 %	0.38	100 %	2.2 %	0.11	89 %	2.0 %	0.10	105 %	1.5 %	0.08	94 %	4.9 %	0.25
	R5	96 %	2.1 %	0.11	102 %	4.1 %	0.21	106 %	2.0 %	0.10	81 %	1.1 %	0.06	85 %	1.9 %	0.10	102 %	3.6 %	0.18
	R6	104 %	1.6 %	0.08	92 %	3.2 %	0.16	97 %	1.7 %	0.09	80 %	1.5 %	0.07	84 %	1.3 %	0.06	91 %	3.7 %	0.19
1000 µg/L	R7	120 %	1.2 %	0.07	96 %	2.3 %	0.14	107 %	2.6 %	0.16	95 %	2.2 %	0.14	105 %	1.7 %	0.11	102 %	1.7 %	0.10
	R8	106 %	1.4 %	0.09	110 %	3.5 %	0.22	116 %	1.2 %	0.08	91 %	2.2 %	0.14	90 %	2.0 %	0.13	119 %	2.3 %	0.15
	R9	116 %	0.9 %	0.05	101 %	2.0 %	0.13	109 %	1.7 %	0.11	88 %	2.9 %	0.18	92 %	1.3 %	0.08	104 %	2.2 %	0.14

Table VII

Intermediate precision values for all compounds at three spiking levels

Spiking µg/L	Sample	Acesulfame-K			Aspartame			Cyclamate-Na			Saccharin			Stevioside			Sucralose		
		Recovery	RSD	HorRat	Recovery	RSD	HorRat	Recovery	RSD	HorRat	Recovery	RSD	HorRat	Recovery	RSD	HorRat	Recovery	RSD	HorRat
50	W1	91 %	13.2 %	0.53	114 %	9.6 %	0.38	97 %	11.3 %	0.45	103 %	5.0 %	0.20	70 %	18.5 %	0.74	101 %	20.5 %	0.82
250	W2	108 %	11.3 %	0.46	82 %	10.0 %	0.40	80 %	6.3 %	0.25	112 %	5.1 %	0.20	61 %	26.4 %	1.06	101 %	23.5 %	0.94
1000	W3	106 %	4.5 %	0.18	83 %	18.1 %	0.73	98 %	2.7 %	0.11	88 %	6.9 %	0.28	81 %	7.6 %	0.31	85 %	12.5 %	0.50
50	Ro1	89 %	14.1 %	0.72	113 %	7.7 %	0.40	95 %	11.5 %	0.59	96 %	3.5 %	0.18	93 %	19.7 %	1.01	79 %	21.1 %	1.08
250	Ro2	101 %	9.4 %	0.48	81 %	6.4 %	0.32	84 %	9.6 %	0.49	108 %	4.0 %	0.20	84 %	21.6 %	1.10	118 %	16.5 %	0.84
1000	Ro3	101 %	3.6 %	0.18	89 %	6.9 %	0.36	91 %	3.3 %	0.17	78 %	4.3 %	0.22	83 %	10.7 %	0.55	91 %	7.9 %	0.40
50	R1	93 %	8.2 %	0.52	119 %	7.7 %	0.49	101 %	13.3 %	0.84	101 %	3.6 %	0.23	98 %	18.8 %	1.19	78 %	17.7 %	1.12
250	R2	109 %	6.1 %	0.38	84 %	8.8 %	0.55	92 %	7.7 %	0.48	117 %	4.1 %	0.26	92 %	20.0 %	1.26	120 %	15.4 %	0.97
1000	R3	111 %	3.7 %	0.23	100 %	4.4 %	0.27	100 %	2.7 %	0.17	86 %	3.7 %	0.23	91 %	9.3 %	0.59	107 %	4.8 %	0.30

within the accepted reference criteria (Commission Regulation (EC) No 401/2006). Other publications do not mention any RSD% values for intermediate precision or reproducibility on which a comparison could be derived.

Recovery

Since the data was obtained exclusively from spiked samples it was possible to use the same data to

evaluate the recoveries. The recovery was maintained roughly constant for all the conditions tested. This behaviour is depicted in Figure 4.

98% of the 162 recovery measurements lie within the 70% to 120% recovery range (SANTE/12682/2019, 2020) with the exception of two values above (1.2% of the values) and two values below (1.2% of the values). The values are randomly distributed in this range (Figure 4).

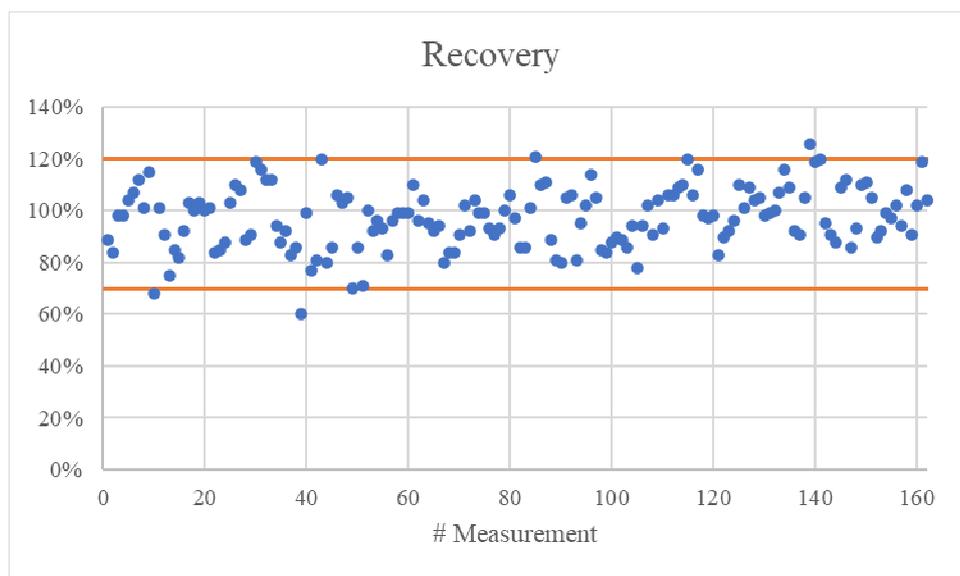


Figure 4. Dispersion of the recovery values obtained for the data of Table VI.

CONCLUSIONS

The method herein presented is applicable to wine and takes into account the high variability of characteristics that can be found. The developed method allows fast detection and quantitation of several sweeteners in the same run with minimal sample preparation steps giving high throughput while achieving a good method performance and is suitable to production and quality control laboratories

During validation the lowest LOD value obtained was 0.002 mg/L which is shared by sodium cyclamate, saccharin and stevioside in the white wine calibrations and by stevioside in the rosé calibration. The LOD range extends up to 0.014 mg/L for sucralose in the white wine calibration. LOQ values extend from 0.005 mg/L for stevioside in white and rosé wines to 0.048 mg/L for sucralose in the white wine calibration. Also, the method was validated with a linearity range from 50 µg/L to 1000 µg/L for all the compounds.

If the method performance becomes an issue there are several aspects that may be a target of improvement, namely the dilution of samples that

fall outside the calibration range, the employment of additional sample preparation steps such as dilution, relying on the instrument sensitivity, sample clean-up and extraction, or even perform the calibrations with the matrix that is being evaluated.

Future work might consist of the inclusion of additional sweeteners in the method and extend the validation to those compounds.

CONFLICTS OF INTEREST: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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