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IDENTIFYING SYNTHETIC SWEETENERS FROM WINE BY UPLC

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Abstract

According to the wine quality and production conditions, the legislation in force states that masking defects and modifications of the wines by additions that determine changes in their taste, aroma and natural composition, can be a counterfeit product. This type of fraud is sanctioned according to the law. An example of these additives are synthetic sweeteners which, although they are forbidden, may be found in wines. Synthetic sweeteners consumption may affect the consumer health, and also the consumption of fraudulent sweetened wines can be a real danger for them. To avoid all of these inconveniences for consumers and for combating fraud in the wine sector, we can highlight the frequency of synthetic sweeteners in bottled wines with a suitable liquid chromatography method. This method establishes how to identify and quantify three of the most commonly used synthetic sweeteners (acesulfame K - E950, aspartame - E951 and saccharin - E954) that can be found in the wine matrix by ultra-performance liquid chromatography (UPLC) with UV detection. The method has selectivity, detection limit and quantification limit, linearity, precision (repeatability and reproducibility), accuracy (bias and recovery).

Key words*: wine, synthetic sweeteners, UPLC, quality, food safety.*

INTRODUCTION

Food and beverage industry has long sought to align itself with international standards on guidelines and regulations for added sugar intake from the World Health Organization. Consumers who want a sweet taste without adding energy can choose to use an artificial sweetener based on their personal taste preference and the intended use, like cooking or table top use (Chakraborty and Das., 2019). World Health Organization recommends a level up to 5-10% added sugar, fact that leads to increased use of non-caloric sweeteners (WHO, 2015). Synthetic sweeteners are in food industry since 1800's and they were often a controversial topic. The addition of non-caloric sweeteners (NNSs) to certain products raises the problem of cumulative effect of additives as well as achieving Acceptable Daily Intakes (ADIs). All over the world acesulfame K and aspartame have been the most frequently evaluated sweeteners and data from different studies showed that the intake was reduced below the level of toxicological concern. The main interest is the potential association between low/no-caloric sweeteners and population health, with special attention on changes in gut microbiota, weight management and obesity, cardiometabolic health and diabetes (Martyn et al., 2018; Reid et al., 2016; Rogers et al., 2016). Research is focused nowadays on the metabolic effects of non-nutritive sweeteners intake. The effect on gut microbiota, that can trigger glucose intolerance (Palmnas et al., 2014; Suez et al., 2014) and the interaction with novel sweet taste receptors discovered in non-taste tissues including the gut and the pancreas, which can influence insulin secretion (Jang et al., 2007; Corkey, 2012) are mentioned in many studies proving that NNSs cause metabolic disorders in human subjects (Suez et al., 2014; Jang et al., 2007; Corkey, 2012; Brown et al., 2009). As well, data from five different mammalian species (human, rats, mice, pigs, etc.) showed that NNSs can be metabolically active (Corkey, 2012; Moran et al., 2010; Mitsutomi et al., 2014). There is clearly that the old concept that

NNSs are invariable metabolically inert is no longer true (Pepino, 2015). Divergent regulations maintain a wide debate on the impact of sweeteners on food safety, consumer's health and people lifestyle (Carocho et al., 2017).

Usage of sweeteners is regulated according to their potential toxicological effects, proposed applications and the level of use in foodstuffs. The exposure of the population to these substances can be monitored by assessing the concentration of the sweeteners in the product and the consumption data of that product (WHO, 2015; D. Martyn et al. 2018). Liquid and gas chromatographic methods were developed for artificial sweeteners determination but because these compounds show low volatility, the last ones were almost abandoned. Several methods based on RP-HPLC with UV or DAD detectors have been reported for determination of aspartame, saccharin, cyclamate, acesulfame K and stevioside in different foods like beverages, canned fruits, ice cream and other dairy desserts (Shah and Jager, 2017). The main problems in developing an HPLC-UV method for NNSs are their different chemical structure and the sample matrix effect. Partially these disadvantages may be overcome when using HPLC coupled with mass spectrometry (MS/MS). Also, with this type of method higher specificity and sensitivity may be achieved. Some HPLC ESI-MS/MS methods were developed, both in negative ion mode (Zygler et al., 2011) and in multiple reaction monitoring mode (Lim et. al, 2013).

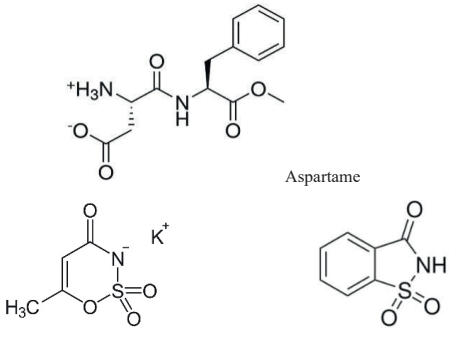

Figure 1. Chemical structure of three artificial sweeteners analysed in this study

European legislation regulates the utilization of nine artificial sweeteners in specified foodstuffs. The most commonly NNSs in foods are: acesulfame K (E950), aspartame (E951) and saccharin and its Na and Ca salts (E954) (Figure 1).

The aim of present study was to find a reliable method for analysing acesulfame K, aspartame, and saccharin in wine using UPLC-UV and to validate it. Method parameters were assessed as follows: selectivity, linearity, sensitivity, accuracy, repeatability, reproducibility, limits of detection, limits of quantification, linear range and recoveries. After validation, the method was used to analyse these three artificial sweeteners in 20 wine samples.

MATERIALS AND METHODS

Materials

For this study the analytical standards of acesulfame K (ACS-K) (6-methyl-1,2,3-oxathiazin-4(3H)-one 2,2-dioxide potassium salt), aspartame (ASP) (N-(L-α-aspartyl)-L-phenylalanine methyl ester) and sodium saccharin (SAC) (2,3-dihydro-3-oxobenzisosulfonazole sodium salt) were purchased from Supelco. Tetrabutylammonium hydrogen sulphate (97%) and anhydrous sodium acetate were supplied by Sigma-Aldrich, as well as the HPLC grade methanol.

Bottled wine samples were purchased from local markets. Red, rosé and white wines classified as dry, medium dry, medium sweet and sweet, were analysed by the presented method. The samples were stored at 4-6°C until analysis.

Standard solutions and samples preparation

Stock standard solutions were prepared in 10% methanol with the following concentrations: 2500 mg/L for ACS K, 4000 mg/L for ASP and 1000 mg/L for SAC. These stock solutions were used in different volumes for each of them to prepare a mixed working standard solution containing 10 mg/L ACS K, 40 mg/L ASP and 10 mg/L SAC. For obtaining the calibration curve, five standard levels were prepared by diluting the mixed working standard solution with ultrapure water. Stock standard solutions and mixed working standard solution were stored at 4-6°C prior to use.

Wine samples were sonicated in order to remove the possible dissolved gases, then they were properly diluted with ultrapure water. After dilution, the samples were filtered using Millex-

HV syringe filter with 0.45 µm pore size PVDF membrane and placed in an UPLC vial for instrumental analysis.
The fortified test samples were prepared by spiking at the level of 40 mg/L ACS K, 60 mg/L ASP and 40 mg/L SAC.

Chromatographic method
In order to identify and quantify the three synthetic sweeteners in wines by liquid chromatography method, we used Waters Acquity UPLC equipment (with binary solvent manager, heater/cooler sample organizer, thermostatic column compartment) with UV detector. The separation was performed with a Luna Omega C18 column (1.6 µm, 100Å, 2.1 mm x 100 mm), at 22°C, by isocratic elution with 0.2 mL/min flow rate. The mobile phase consisted in a mixture of two solvents: 400 mL of 5 mM tetrabutylammonium hydrogen sulphate and 6.1 mM anhydrous sodium acetate solution prepared in ultrapure water mixed with 171 mL 100% methanol. The solvents were degassed before using. The injection volume was 2.0 µL and the run time was 15 minutes. Artificial sweeteners detection was performed at 220 nm. Data were collected and processed using Empower 2 software.

RESULTS AND DISCUSSIONS

The described chromatographic method was applied for quantification of the three synthetic sweeteners. After diluting the stock solutions, each analyte was injected in turn, in order to determine their sequence. All the three sweeteners were identified in the chromatogram obtained for the mixed standard solution, in the following order: ASP (5.98 min retention time), ACS K (6.95 min retention time) and SAC (10.01 min retention time). The analysis revealed a good separation of the three compounds, indicated by resolution and peaks shape and symmetry (Figure 2). Taking into account the retention times and peak width, the calculated values for resolution were 28.7 for ASP, 2.3 for ACS K and 6.5 for SAC.
The method was validated to demonstrate that its performance characteristics are adequate to using for intended purpose (Barwick et al., 2014). There have been established and confirmed specific validation parameters, like selectivity, limit of detection and limit of quantification, working range, analytical sensitivity, trueness, precision, measurement uncertainty, ruggedness (Barwick et al., 2014).
Selectivity is ability of a method to measure the differences of analysis in the presence of other compounds that behave similarly (Rusea, 2016). Absence of interferences and clear identification of each compound were registered. The peaks are completely resolved, with no overlapping (Figure 2).
Limit of detection (*LOD)* of an individual analytical procedure is the smallest amount of analyte in test sample that can be detected, but it doesn't mean that can be quantified as an exact value (Rusea, 2016). A signal-to-noise ratio of 3 was evaluated for LOD.

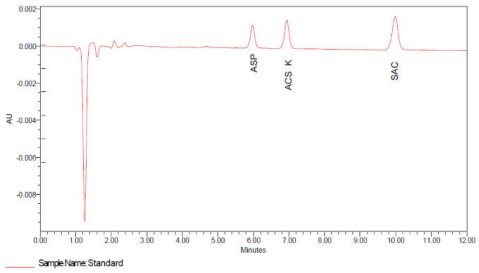

Figure 2. Chromatogram of mixed standard solution

The determined values of LOD were 1.4 mg/L for ASP, 0.3 mg/L for ACS K and 0.2 mg/L for SAC.
Limit of quantification (LOQ) is the smallest amount of analyte in test sample that can be quantitatively determined with acceptable repeatability and accuracy (Rusea, 2016). Evaluation was made considering three times LOD values. The results obtained for LOQ were 4.2 mg/L for ASP, 0.9 mg/L for ACS K and 0.6 mg/L for SAC.
Working range is the interval between lower and upper concentration of analyte in a sample for which the analytical procedure has been adequate (Rusea, 2016). Generally, it is much wider than the linear domain. Our working range was established between 4.0 mg/L and 40.0 mg/L for ASP, 1.0 mg/L and 40.0 mg/L for ACE K and 0.5 mg/L and 40 mg/L for SAC.
Linearity is the ability of a method to provide results directly proportional to the analyte concentration on an established domain (Rusea, 2016). Quantitative analysis were performed

using external calibration method. Calibration curve was obtained with standard solutions with five levels of concentration, with three injections per each level. The correlation coefficient was higher than 0.99 for all the three artificial sweeteners. The obtained values of r^2 were 0.9974 for ASP, 0.9959 for ACS K and 0.9935 for SAC.

Analytical sensitivity is the modification in response of a measuring instrument divided to the corresponding change of the stimulus (Rusea, 2016). It expresses the ability of a method to record small variations in concentration of a certain analyte and we confirmed that this method has analytical sensitivity.

Trueness or accuracy of an analytical procedure express how close the experimental value is to the true value. It indicates the concordance between average value of a set of results and an accepted reference value (Rusea, 2016). Measure of trust is expressed in terms of bias which represents a total systematic error. Our bias is 6.9% for ASP, 4.5% for ACS K and 6.8% for SAC.

Repeatability or precision means the approaching results from a series of measurements obtained from different aliquots of the same homogeneous samples, under the same conditions (Rusea, 2016). It is expressed as relative standard deviation (RSD%) and it is a component of measurement uncertainty. Repeatability was assessed by injecting 6 times in a row three levels of the mixed standard solution. The average values obtained for RSD were 1.6% for ASP, 2.1% for ACS K and 1.9% mg/L for SAC.

Reproducibility is when repeatability is made by another analyst, or using another equipment (with the same configuration), or in another laboratory, but following the same analytical procedure for analysing the same sample. For this study, the reproducibility was assessed by analysing the same three levels of mixed standard solution, 6 times in a row by two analysts. The registered average values for RSD were 8.4% for ASP, 7.3% for ACS K and 4.4% mg/L for SAC.

Recovery is the percentage of the real concentration of a substance recovered during the analytical procedure. It is a measure of method efficiency for detecting the all analyte (Rusea D., 2016). It is expressed as a ratio between response obtained for the samples extracted at three concentrations of analyte and the response measured without the extraction step. Recovery test was performed analysing wine samples fortified with 40 mg/L ACS K, 60 mg/L ASP and 40 mg/L SAC. The determined recovery values were 92% for ASP, 90% for ACS K and 101% SAC.

Measurement uncertainty is a parameter associated with the measurement result that characterizes dispersion of attributed values to the measurement. Uncertainty means evaluation of sources of errors at each stage and estimation of associated uncertainty (Rusea D., 2016) and it is an essential component of the validation process. The global uncertainty it is based on available data from validation method, internal quality control and comparison tests. For this method we established a measurement uncertainty up to 8%.

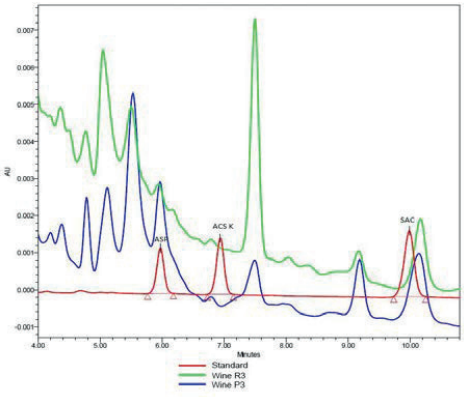

Figure 3. Overlay chromatogram of two positive wine samples for aspartame (R3 and P3) and mixed standard solution

The described method was used for assessing the presence and the content of the three NNSs in 20 wine samples from Romanian market. According to the obtained data, two samples were positive for aspartame, one red dry wine (R3) with 4.701 mg/L and one rosé sweet wine (P3) with 22.858 mg/L. Overlaying the chromatograms of the two samples and the one of the standard, it is obvious the presence of aspartame and the absence of the other two sweeteners (Figure 3). Acesulfame K and saccharin were not detected in any sample.

CONCLUSIONS

The presented UPLC-UV method is suitable for determination of the concentration of illegally added acesulfame K, aspartame and saccharin in wine. The reliability of this method is assured by the obtained values for the validation parameters. The method is rather simple, with no need of complicated sample preparation.

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