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Simultaneous extraction and analysis of preservatives and artificial sweeteners in juices by salting out liquid-liquid extraction method prior to ultra-high performance liquid chromatography.

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Abstract

A novel and fast salting out liquid liquid extraction method was developed for simultaneous determination of food additives with different polarities in juices. Chromatographic separation was achieved in less than 6 min using Acquity UPLC BEH C 18 (100 mm x 2.1 mm d.i. x 1.7 µm) column with ammonium acetate with 0.01 % of trifluoroacetic acid as eluent A and acetonitrile as eluent B at a flow rate of 0.2 mL min^{-1}. The main factors affecting the extraction efficiency were optimized. The method was validated applying accuracy profile based on total error. The extraction recoveries ranged from 84.97 to 122 %. Relative standard deviation ranged from 1.24 to 7.99 % for intraday assay and from 1.69 to 9.16 % for intermediate precision. The limits of detection for five food additives were from 0.3 to 1.42 µg mL^{-1}. The method was successfully applied to 47 samples of juices from nine brands.

Keywords: Food additives, salting out liquid-liquid extraction, RP-UPLC, accuracy profile, juices.

Chemical compounds studied in this work

Potassium acesulfame (PubChem CID: 11074431); Sodium saccharin (PubChem CID: 23696271); Aspartame PubChem CID: 134601; Sodium benzoate (PubChem CID: 517055); Potassium sorbate (PubChem CID: 23676745).

1. Introduction

Food additives include preservatives such as benzoic acid, sorbic acid and their salts which are added to foods to inhibit bacterial and fungal growth in order to improve food shelf life (Boyce, 1999). Sweeteners such as, acesulfame, saccharin and aspartame are synthetic, non-metabolized, non-nutritive and non-caloric dietetic sweeteners with sweetness hundreds of times stronger than that of sugars. They are widely used in food industries to improve the sweet taste and to replace sugars in foods for reducing caloric intake, as well as for diabetics for whom sugar restriction is recommended (Bergamo, Da Silva & De Jesus, 2011).

Current national, international legislation and safety agencies of different countries regulate the additives and their maximum amount or limit that can be added to food. As stated by Algerian food additives legislation, sweeteners are authorized in juices and nectars or concentrated fruit nectars at concentration of 350, 600 and 80 µg mL^{-1} for acesulfame, aspartame and saccharin, respectively. However, benzoic and sorbic acids are forbidden (Ministère du commerce Algérien, décret exécutif n°12-214. 2012). Joint FAO/MOS Expert Committee on Food Additives (JECFA) have set the acceptable daily intake (ADI) of these compounds at the range 0-15, 0-5, 0-40, 0-5 and 0-25 mg kg^{-1} for acesulfame, saccharin, aspartame, benzoic acid and sorbic acid, respectively (WHO, 1999). Otherwise, potential and harmful risks to human health can raise when these compounds are added at high amount to food. Some studies suggested that very high intake of benzoic acid or its salts could cause adverse health effects such as metabolic acidosis, hyperpnoea and convulsions (WHO, 1997). In sensitive persons, even consumed at concentration lower than 5 mg/kg of body weight per day, benzoic acid can cause non-immunological contact reactions (WHO, 2000). It has been proved that benzoic acid in products with high content of ascorbic acid together with transition-metal catalyst, reacts and produce benzene considered as carcinogenic agent (Cakir & Cagri-Mehmetoglu, 2013).

According to EFSA CEF Panel (2011), after oral intake, benzoates are rapidly and fully absorbed by the gastrointestinal tract, metabolized primarily in the liver, and excreted in the urine as glycine

conjugates of benzoic acid derivatives, mainly as hippuric acid. Sorbic acid is harmless and it is absorbed and mainly excreted or expired as carbon dioxide (WHO, 1974). According to JECFA (WHO, 1993), saccharin is not metabolized and it is suspected to induce bladder cancer in male rat, which is not relevant to human. Pharmacokinetic studies show that acesulfame is completely absorbed, not metabolized and rapidly excreted unchanged and no adverse effects are associated to acesulfame (WHO, 1991). Aspartame is metabolized in the body into aspartic acid, phenylalanine and methanol and high level of phenylalanine in plasma is known to cause developmental toxicity in humans to individuals with inherited metabolic disorder called phenylketonuria (EFSA ANS Panel, 2013). Moreover, as stated by Swithers-Susan (2013), other harmful effects can be associated to artificial sweeteners with negative health outcomes such as risk of weight gain, metabolic syndrome, type 2 diabetes, hypertension and cardiovascular disease. Therefore, the use of preservatives and artificial sweeteners in foodstuffs must be monitored.

Recently, several analytical methods were employed for separation and quantification of food additives. High or ultra-high performance liquid chromatography (HPLC or UPLC) coupled to ultraviolet detector (Diogo, Silva, Pena & Lino, 2013; Lino et al., 2010), diode array detector (DAD) (Dias, Meinhart, Pane, Ballus & Godoy, 2015) have been proposed. In addition, tandem mass spectrometry (MSn) (Chang & Yeh, 2014) were the main used techniques. Other techniques were also used and include spectrophotometry UV with PLS-2 method (Cantarelli, Pellerano, Marchevsky & Camina, 2009), capillary electrophoresis (CE) with capacitively coupled contactless conductivity detection (CE-C^4D) (Bergamo et al., 2011) and micellar electrokinetic chromatography (MEKC) coupled to UV and DAD (Boyce, 1999;).

Sample pretreatment and cleanup is a mandatory step in food samples in order to remove the matrix interference prior to the chromatographic determination. Various sample preparation and simultaneous extraction ~~processes~~ of these five compounds have been reported in literature such as, centrifugation, microfiltration and dilution (Diogo, Silva, Pena & Lino, 2013; Lino et al., 2010). Nevertheless, the importance of these techniques cannot be underestimated, as not all errors that

occur in this step can be corrected, even by the best separation or detection method (Szultka, Pomastowski, Railean- Plugaru & Buszewski, 2014). Microextraction method such as dispersive solid-phase extraction (dSPE) using ethylenediamine or tetraethylynepentamine functionalized Fe_3O_4 magnetic polymer (TEPA MP) and (IEPA MP) were developed (Chen, Zhao, Shen & Jin, 2012; Zhao, Cai, Chen, Pan, Yao & Jin, 2013). These last extraction techniques are tedious, time consuming and expensive, because of the preparation and the synthesis of the TEPA MP and IEPA MP, which use expensive reagents, and their characterization with transmission electron microscopy, which cannot be available in every laboratory. Moreover, these techniques are used to remove natural pigments, organic acids and sugars from the matrix, where a limiting factor can be the sorption of the analytes on these compounds, and thus, the low recoveries.

Non-polar water-immiscible organic solvents used in liquid phase microextraction (LPME) have low dielectric constant and they are relatively poor for extraction of polar compounds. More-polar water-miscible solvents such as acetone, acetonitrile, ethanol and isopropanol, that provide solubility for polar to non-polar compounds, cannot be used for conventional liquid liquid extraction (LLE) or LLME method. However, if the solvent is generated *in situ* in the aqueous solution and a phase separation further occurs, two-phase system is obtained upon the addition of an appropriate quantity of an electrolyte, such as a salt, that decrease the miscibility of two mixed liquids (Tabata, Kumamoto & Nishimoto, 1996). Then, the analytes can move selectively from the aqueous phase into the polar organic phase. This process is called salting out and it is applied in salting out liquid liquid extraction (SALLE) or extraction by demixture (Nerín, Polo, Salafranca & Cacho, 1996). Recently, this technique was applied as a simple, fast, economical, green and benign extraction/cleanup method for the preparation of various samples and extraction of different compounds such as mycotoxins, antibiotics, pesticides, drugs, polyphenolic compounds and metals from different matrices such as water, biological fluids and food (Magiera & Kwietniowska, 2016).

The objective of this study is the optimization and validation of fast and efficient salting out liquid liquid extraction (SALLE), coupled to UPLC-UV, for the simultaneous extraction and

determination of food additives with different polarities from the same aliquot of sample. Univariate optimization method was used to optimize the main and effective parameters affecting the extraction, to enhance the efficiency of the method. The optimized method was validated using accuracy profile and applied to the analysis of 47 samples of juices ~~beverage samples~~ to check if these five additives (acesulfame, saccharin, aspartame, benzoic and sorbic acids) were legally used and within the maximum permitted levels.

To our knowledge, there are no literature describing the simultaneous extraction and analysis of sweeteners and preservatives with the application of SALLE method. The results based on the application of this method show that the method is simple, exhibits excellent applicability, rapid, cheap, environmentally friendly, and very suitable for extraction of food additives with different polarities. As in SALLE method the extraction solvent is generated in situ, handling of etraction and enrichment factors can be very high, and the complexity of the extraction is considerably reduced.

2. Materials and methods

2.1. Reagents

Potassium acesulfame (≥ 99 %), sodium saccharin (≥ 99 %), and aspartame (≥ 99 %), Sodium benzoate (≥ 99 %) and sorbate potassium (≥ 99 %) were obtained from (Sigma Aldrich, Germany). Acetonitrile, acetone, anhydrous ethanol, isopropanol, methanol (HPLC or LC MS grade), hydrochloric acid (HCl, 37%), sodium chloride (NaCl, ≥ 99.5) and sodium sulphate (Na_2SO_4, ≥ 99 %) were obtained from (Scharlau, Spain). Ultrapure water used for samples preparation through the work was purified from Millipore system (Milli-Q plus 185). Ammonium acetate (CH_3COONH_4, ≥ 98%), magnesium sulphate heptahydrate (Mg SO_4 7 H_2O) and ammonium chloride (NH_4Cl, ≥ 99.8 %) were obtained from (Merck, Germany). Ammonium sulphate ($(NH4)_2$ SO_4, 99%) was purchased from (Pancreac Appli Chem Barcelona, Spain) and Trifluoroacetic acid (TFA, 99 %) was purchased from (Fluka, Switzerland). Flow of N_2 (ALPHAGAZI, 99.999% Global purity, Air Liquide Zaragoza-Spain) was used for the evaporation of extraction solvent.

149 *2.2. Preparation of standards*

150 Stock solutions of each compounds were prepared at concentration of 1000 µg mL^{-1} in ultrapure
151 water for acesulfame, saccharin, sodium benzoate and potassium sorbate and in mixture of water:
152 methanol (50: 50 v/v) for aspartame.

153 *2.3. Procedure of method validation*

154 According to the guidelines of the "French Society of Pharmaceutical Sciences and Techniques"
155 (SFSTP) for the validation of the analytical method using accuracy profile methodology requires
156 two different standard solutions namely calibration standards (CSs) prepared without matrix and
157 validation standards (VSs) or quality control samples (QC) prepared with the presence of the
158 matrix. In this work, modified validation experimental protocol V2 with the addition of two levels
159 to CSs and VSs was used. Therefore, three series of five working solutions with two replicates for
160 CSs and three replicates for VSs were prepared each day.

161 This approach is based on β expectation tolerance interval that summarizes in a single graph the
162 total error measurement for the sum of the bias and standard deviation of intermediate precision
163 (Hubert et al., 2007). A procedure can be qualified as acceptable and gives accurate results if the
164 difference between every recovered concentration (x) of a sample and its true concentration is
165 inside the acceptance limits ± λ settled by the analyst

166 Different validation criteria as response function, trueness, precision, accuracy, linearity, lower
167 and upper limit of quantification (LLOQ, ULOQ) and limit of detection (LOD) were used in order
168 to validate the method.

169 *2.4. Optimization of RP-UPLC conditions*

170 Ultrahigh performance liquid chromatography analysis was performed on an Acquity UPLC BEH
171 C 18 (100 mm x 2.1 mm d.i. x 1.7 µm) analytical column coupled to Acquity UPLC BEH C 18 1.7
172 µm guard column. Analytes separation performed with gradient elution using ammonium acetate
173 buffer 2.5 mmol L^{-1} acidified with trifluoroacetic acid at 0.01 % (v/v) as eluent A and acetonitrile as
174 eluent B. The separation was achieved with optimized gradient as follows: 7 % B (0- 2 min), 35 % (3

175 min), 10 % (4 min), 10 % (4.01 min) and 7 % (5.5 min) at flow rate of 0.2 mL min $^{-1}$. In order to clean
176 the column, 100 % of B was used for 1.5 min, then 7 % of B for 2 min to re-equilibrate and come
177 back to initial condition. The column temperature was kept at 40 °C and the injection volume was 10
178 µL. All the analytes were monitored and detected at wavelength of 210 nm and were eluted in less
179 than 6 min in the order: acesulfame, saccharin, aspartame, benzoic and sorbic acids.

180 *2.5. Collection of the samples and extraction method*

181 Forty-seven juice beverages products from nine different brands were collected from different
182 supermarkets area in Tizi Ouzou city (North of Algeria). All the samples were stored under
183 refrigeration conditions (4 °C) until analysis.
184 The samples were centrifuged twice, 20 min at 4000 RPM each time, and then diluted 20 times to
185 reduce matrix effect, adjusting the pH to 3 with HCl solution (0.7M, v/v).
186 For SALLE method, 3 mL of the prepared sample or mixture of standards were placed in 15 mL
187 screw capped polyethylene test tube containing 1.7 g of ammonium sulphate and the mixture was
188 shaken until the complete dissolution of salt. After that, 0.5 mL of the mixture acetone: ethanol (50:
189 50 v/v) was added using 1 mL micropipette. The mixture was vortexed for 1 min at 3000 RPM then
190 centrifuged for 12 min at 4000 RPM to induce the phase separation. The upper phase was carefully
191 recovered, transferred to 2 mL vial and evaporated under a gentle nitrogen stream at 60 °C to eliminate
192 the organic solvent. To avoid peak overlap, the residue was reconstituted with 1.5 mL of buffer
193 solution of mobile phase, shaken in vortex for 2 min, and injected into UPLC-UV for analysis.

194 *2.6. Data analysis*

195 All the statistical analysis were calculated using the back-predicter and E-noval V3.0 softwares
196 (arlenda, Liège, Belgium).

197 **3. Results and discussion**

198 *3.1. Optimization of the method*

Fruit juices are complex matrices containing sugars, and fibers ~~flavonols and preservatives~~ among other compounds at low concentration such as proteins. For this reason, sample pretreatment and cleanup is a mandatory step in order to isolate the compounds of interest. In this work, different compounds with different polarities as amino and carboxylic groups were the target. Hence, the simultaneous extraction of these compounds with water-immiscible organic solvents is not an easy task. The low dielectric constants of these solvents and the polar nature of these compounds, which are slightly or very slightly soluble in immiscible water solvent, make the extraction of the mentioned compounds quite poor, mainly for saccharin, aspartame and acesulfame. However, all these compounds are soluble in water miscible solvents with high dielectric constant i.e. ethanol, acetone, acetonitrile and isopropanol. Hence, SALLE using these water miscible solvents was selected for simultaneous extraction of these compounds. These solvents can dissolve in water in any proportion because of "hydrogen bond" interaction. The presence and the increasing amount of ammonium sulphate in aqueous solution, containing ethanol and acetone, leads to the migration of water molecules away from ethanol and acetone molecules to ions of salts and the mass fraction of acetone and ethanol increases in the upper phase (Wang, Yan, Hu, Han & Xu, 2010). Therefore, at saturated concentration of the ammonium sulphate two phases are formed *in situ*.

For this purpose, 3 mL of ultrapure water spiked with standards at concentration of 20 µg mL^{-1} for acesulfame, aspartame, benzoic and sorbic acids and at 4 µg mL^{-1} for saccharin was used for method optimization using univariate optimization method, by varying one factor while keeping all the other factors at constant level. The main factors affecting the extraction efficiency measured as the chromatographic peak area were the nature and the volume of the extraction solvent, the amount of salt and the pH of sample. All the results are the average of triplicate measurements. Optimization of factors influencing the extraction method is discussed below in detail and the optimized results were 0.5 mL for the mixture of ethanol: acetone at (50: 50 v/v) as extraction solvent, 1.7 g/ 3mL of ammonium sulphate used as salting out agent and pH 3.

3.1.1. Selection of extraction solvent type and volume

Selection of extraction solvent is an important consideration and crucial step to control the efficiency of SALLE process. This solvent must be polar, miscible with water in all proportions, with high capability to dissolve the analytes and easily separable from water by adding an electrolyte as salt. The saturated concentration of the salts in the presence of different solvents is different for each compound. Therefore, different tests were performed to determine the saturated concentration of ammonium sulphate in water with achieving a salting out effect in the presence of the tested extraction solvents i.e. (acetone, ethanol, acetonitrile and 2-propanol). These amounts were found and ranged from 1.7 to 2.05 g. In order to select the most appropriate organic solvent, 1 mL of each solvent was tested in the mixture of 3 mL of sample and different ammonium sulphate amounts. The results are shown in fig. 1A. As can be seen, the maximum responses were obtained with ethanol for acesulfame, saccharin and aspartame and with acetone for benzoic and sorbic acids. Therefore, the mixtures of both ethanol and acetone at different ratios of (50: 50; 75: 25 and 25: 75 v/v) were tested. As shown in fig. 1B, the sum of peak areas was identical. However, the ratio (50: 50, v/v) gave the best results for saccharin used at very small concentration in juices products. Therefore, the binary mixture of ethanol: acetone at (50: 50 v/v) was used as suitable extraction solvent.

The volume of extraction solvent was studied at 0.5, 0.8, 1.1, 1.4, 1.6 and 2 mL. The results illustrated in fig. 1C show that as the volume of extraction solvent increases the sum of peak areas for all the compounds decreases and the results are higher for 0.5 mL of extraction solvent. This is because when increasing the volume of extraction solvent, the amount of analytes transferred increases too and more food additives are extracted from the aqueous phase. However, the dilution effect reduces the analyte concentration with high extraction solvent volume. Therefore, 0.5 mL was selected as the optimum volume for the extraction.

3.1.2. Selection the type and the amount of salt

As it is known the salting out for phase separation between organic extraction solvent and the aqueous solution varies with the type of salt added. Therefore, different organic and inorganic

salting out agents other than ammonium sulphate were checked at their saturated concentration of 1.08, 3.7, 1.42 and 1.15 g for sodium chloride, magnesium sulphate heptahydrate, sodium sulphate and ammonium chloride, respectively. The results showed that the best two-phase separation was obtained for ammonium sulphate. For sodium and ammonium chloride, two phases were not obtained. This is probably due to the presence of methanol in the sample used for the preparation of aspartame. Moreover, these salts have low salting out effect because these salts are with monovalent anion and cation (Lu, Hao, Hu, Han, Tan & Yan, 2013). For magnesium sulphate heptahydrate, the two phases were not clearly separated. In the case of sodium sulphate, an important salting out effect was observed mixture of extraction solvent and water was released. This is probably due to the transference of the aqueous phase into the organic solvent. Therefore, ammonium sulphate that gave the best two-phases separation was selected for the rest of the work.

The ionic strength also influences the solubility of the analytes in the sample solution. Therefore, the effect of the concentration of salt on the extraction efficiency was studied with addition of different ammonium sulphate amount, ranging from 1.6 to 1.9 g/ 3 mL. As shows fig. 2, when the concentration of salt increases from 1.6 to 1.7 g, the response for the sum of peak areas increases too. This can be explained by the hydration theory, as when the concentration of salt ions in water increases, more water molecules form preferentially hydration sphere around the salt ions, and fewer water molecules are available to hydrate the organic compounds (Kokosa, Przyjazny & Jeannot, 2009). This is explained by the difference in the acting force between an “ion-water” and an “alcohol-water” pair, leading to separation of organic phase rich in analytes (Wang et al., 2010). However, the response for the sum of peak areas decreases when the amount of salt is added at high concentrations of 1.8 and 1.9 g. This can be explained by the increase of viscosity of the aqueous solution, which result in a difficult mass transfer.

3.1.3. Selection the *pH of sample*

The pH of the sample is a significant factor affecting the extraction efficiency and the transfer of the analytes to the organic phase. To improve the extraction efficiency, the pH of sample should be

277 modified to suppress the ionization of any acidic or basic analytes, considering the pKa of the
278 studied compounds acesulfame (pKa = 2), saccharin (pKa = 2), aspartame (pKa = 3.1, 7.9) benzoic
279 acid (pKa = 4.19) and sorbic acid (pKa = 4.76). At high pH the ionizing abilities for the amino and
280 carboxylic groups of these food additives is very high. The basic compounds will be as neutral
281 molecules, extracted by the organic solvent, and can interfere with the studied compounds. To avoid
282 this drawback, the pH was studied in the range from 1 to 4. As stated by Wang et al. (2010) at lower
283 pH values, higher volume of organic phase is released. Therefore, the dilution effect of extracted
284 compounds will be important. Indeed, the results from fig. 3 show that the satisfactory extraction
285 efficiency was obtained at high pH of 3 and 4. However, RSD % from triplicate assay are from 0.95
286 to 6.85% with an average of 3.11 % at pH 3 and are from 3.86 to 7.39 % with an average of 6.40 %
287 for all the compounds. Thus, the results are better for pH 3, which was selected as optimum for
288 subsequent tests.

289

290 *3.2. Validation of the method*

291 In method validation, usually the linearity of the calibration curve obtained using the simple least
292 squares regression model, $y = ax + b$, is the first parameter to be checked using correlation
293 coefficient (R^2). If this parameter is ≥ 0.99, the simple regression model is considered suitable to
294 predict the unknown amount in the sample. However, it was demonstrated that a simple regression
295 model with a correlation coefficient ≥ 0.99 could not be a reliable indicator of linearity (Shewiyo et
296 al., 2012) and it cannot be suitable due to the possible presence of significant lack of fit (Araujo,
297 2009; Sonnergaard, 2006).

298 Using SFSTP 2006 guidelines for alternative approach of the accuracy profile validation,
299 different linear and nonlinear response functions (linear and quadratic with logarithm, square root,
300 weighted $1/x$ and $1/x^2$ transformation) describing the relationship between quantity (x) and response
301 (y) are tested using CSs data (Hubert et al., 2007 a, b). The response functions were tested and the
302 best one for which the β expectation tolerance intervals included in the settled limits were selected.

Based on the accuracy profile results, several acceptable response functions were obtained for each compound. However, linear regression after logarithm transformation was selected as the simplest one and applied as regression model for all the studied compounds.

The precision of the method evaluated at each level of VSs provides information regarding the random error describing the degree of which data generated from replicate measurements differ from one to another. It was evaluated at two levels of repeatability (intra assay precision) and intermediate precision (inter day precision) by measuring the relative standard deviation. The results are shown in Table 1. The RSD % values obtained were very good and they ranged from 1.24 to 7.99 % for repeatability and from 1.69 to 9.16 % for intermediate precision.

The trueness of the method measured at each concentration level express the difference between the obtained analytical value and the true value accepted as reference. It can be estimated in absolute or relative bias or in term of absolute or relative recovery. In solvent extraction method, relative recovery defined as the ratio of the back-calculated concentrations found in the investigated matrix to those in distillated or deionized water is generally used. The results show that the relative bias is good and ranged between 1.19 and 22.05 %. Moreover, the obtained recoveries are satisfactory and ranged from 84.97 and 122 % for all the compounds.

The linearity of the method was evaluated and determined by fitted linear regression curve obtained by plotting back calculated concentrations of validation standards versus theoretical or introduced concentration and applying the best selected model for each compound. All the regression fitted well with minimal coefficients R^2 value ≥ 0.99, showing the good linearity of the results generated by the SALLE-UPLC-UV method.

Accuracy express the closeness of agreement between the test results and the accepted reference or conventionally true value. The total error is taken into account, which is the sum of trueness (systematic error) represented by the relative bias (%) and precision (random errors) represented by the lower tolerance limit (LTL) and the upper tolerance limit (UTL). The accuracy profiles given in fig. 4 show that 80% of the β expectation tolerances limits for all compounds are inside the

acceptance limits set at ± 30 %. This means that the analytical SALLE-UPLC-UV method is capable of making accurate results over the whole concentration range for all the compounds.

Consequently, the lower and upper quantitation limits (LLOQ and ULOQ) are the extreme values that can be quantified with a defined accuracy and were [2.49-49.87], [0.99-19.98], [2.49-49.79], [5.14, 102.84], [5.02, 100.42] for acesulfame, saccharin, aspartame, benzoic acid and sorbic acid, respectively. As the accuracy profile approach is used for method validation, the LOQ correspond to the LLOQ that can be detected and quantified accurately. The results summarized in table 1 show that the LOQs ranged from 0.99 to 5.02 µg mL^{-1}.

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified as an exact value. In this work, the LOD was calculated using the methodology described by Miller & Miller (1993). This method is based on the computation by series of the Y_{LOD} = Intercept (0 if negative) + 3 x Residual SD obtained by ANOVA. Using the selected regression model, the back-calculation will give the X_{LOD} for each series. The mean of the different back-calculated X_{LOD} will give the LOD of the procedure. The results summarized in table 1 show that the LODs ranged from 0.3 to 1.42 µg mL^{-1}.

3.3. Analysis of real samples and occurrence of additives

The developed method was successfully applied for the determination and quantification of three artificial sweeteners and two preservatives in 47 juices products belonging to nine different brands from Algerian market. The results from all analyzed samples are summarized in table 2. As regards to acesulfame, saccharin and aspartame, fortunately, none of these compounds were found in any analyzed product and these results are in accordance with the Taiwan study of Chang et al. (2014). However, all the other studies found and quantified at least one sweetener in the analyzed samples of juices (Bergamo et al., 2011; Dias et al., 2015; Diogo et al., 2013; Lino et al., 2010).

Preservation technology such as pasteurization, aseptic packaging and the high acidic character (pH 3- 4) of fruit juice can be used for protection of fruit juices against the growth of microorganisms (Cakir & Cagri-Mehmetoglu, 2013). It is important to highlight that benzoic and

sorbic acids or their salts are forbidden in juices and nectars by Algerian food additive legislation (Ministère du commerce Algérien, décret exécutif n°12-214. 2012). However, potassium sorbate was found declared in the label claim of two brands of analyzed samples but their concentrations were not given.

The results of the analyzed samples show that both benzoic and sorbic acids were detected and quantified separately in 12 samples (25.53%) and simultaneously in 6 samples (12.76%). The mean concentrations measured were 63.03 ± 14.23 and 65.69 ± 21.76-µg mL^{-1} ranging from 36.34 to 102.09 and 32.34 to 118-µg mL^{-1} for benzoic and sorbic acids or their salts, respectively and a maximum of 171.6 µg mL^{-1} for the sum of the two compounds when detected simultaneously. The results were not in accordance with the guidelines of Algerian standard legislation (Ministère du commerce Algérien, Décret exécutif n°12-214. 2012) as the use of these additives in juices is forbidden ; however, they were below or did not surpassed the allowed limit of 1000 µg mL^{-1} set by Codex Alimentarius (2016).

The results found in this work are lower for benzoic acid and very similar for sorbic acid to those found by the Portuguese study of Mota et al. (2003). Moreover, as in our study, the researchers did not detect exceeding levels of benzoic or sorbic acids in fruit juices.

The study of Cakir & Cagri-Mehmetoglu (2013) showed that only one juice sample contained benzoic acid at 181.4 µg mL^{-1}. Moreover, benzoic and sorbic acids were not detected in fruit juices, and only traditional soft drinks or soft drinks based on mineral water had those additives (Diogo et al., 2013). The results found were not too high to be dangerous for the consumers. The concentration levels found could be from the indigenous and natural content of fruits (Davidson, et al., 2001) or resulted from the contamination of fruits used in the preparation of the samples of juices by air, rain, soils and water which could contain benzoic acid (Javanmardi et al., 2015).

4. Conclusion

In this study, a simple, fast, economical, green and benign extraction/cleanup method namely SALLE coupled to UPLC-UV was applied for routine monitoring and quantitative determination of

five food additives with wide range of physicochemical properties namely acesulfame, saccharin, aspartame, benzoic and sorbic acids in samples of juices. This method reduces the consumption of the extraction solvent and sample volumes to a very low level compared to the classic extraction methods such as LLE and SPE. The method was optimized using univariate methodology and validated using accuracy profile approach based on the total error. Under the optimum extraction conditions, the optimized method provides suitable trueness, precision, extraction recoveries, linearity, accuracy and sensitivity in a short time. The method was successfully applied to the analysis of samples of juices to ensure a good quality of products and compliance with additive legislation, in order to avoid the effects of these sweeteners and preservatives on people's health if used at high concentrations. The results show that only benzoic and sorbic acids were detected and quantified in the analyzed samples and there are no problems regarding the concentrations found which were not of concern for consumer health.

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Fig. 1. Influence of (A) Extraction solvent type, (B) Ratio of acetone and ethanol and (C) Volume of extraction solvent on SALLE efficiency. Extraction conditions: (A) extraction solvent volume, 1 mL; ammonium sulphate amount, 1.7-2.05 g, 1.7-2.05 g / 3mL and pH, 2; (B) extraction solvent volume, 1 mL ; salt concentration, 1.7 g/ 3 mL and pH, 2; (C); Extraction solvent, ethanol: acetone (50, 50 v/v); salt concentration, 1.7 g/ 3 mL and pH, 2.

Fig. 2. Influence of salt amount for SALLE efficiency. Extraction conditions: Extraction solvent, ethanol: acetone (50, 50 v/v); Solvent volume, 0.5 mL and pH, 2.

Fig. 3. Influence of pH on SALLE efficiency. Extraction conditions: Extraction solvent, ethanol: acetone (50, 50 v/v); Solvent volume, 0.5 mL; salt amount, 1.7 g/ 3 mL.

Fig. 4. Schematic representation of the accuracy profiles obtained for all the compounds. (A) Acesulfame; (B) Saccharin; (C) Aspartame; (D) Benzoic acid and (E) Sorbic acid. The dotted lines are the lower and upper acceptance limits set at 30 %, the dashed lines are the lower and upper β-expectation tolerance limits and the continuous line is the relative bias.

Tables

Table 1: Results of analytical performances of SALLSE- UPLC-UV method for the quantification of the five food additives in juice sample (p= 3, m= 5 and n= 3).

Table 2: Results of the occurrence and concentration of food additives in real commercial samples

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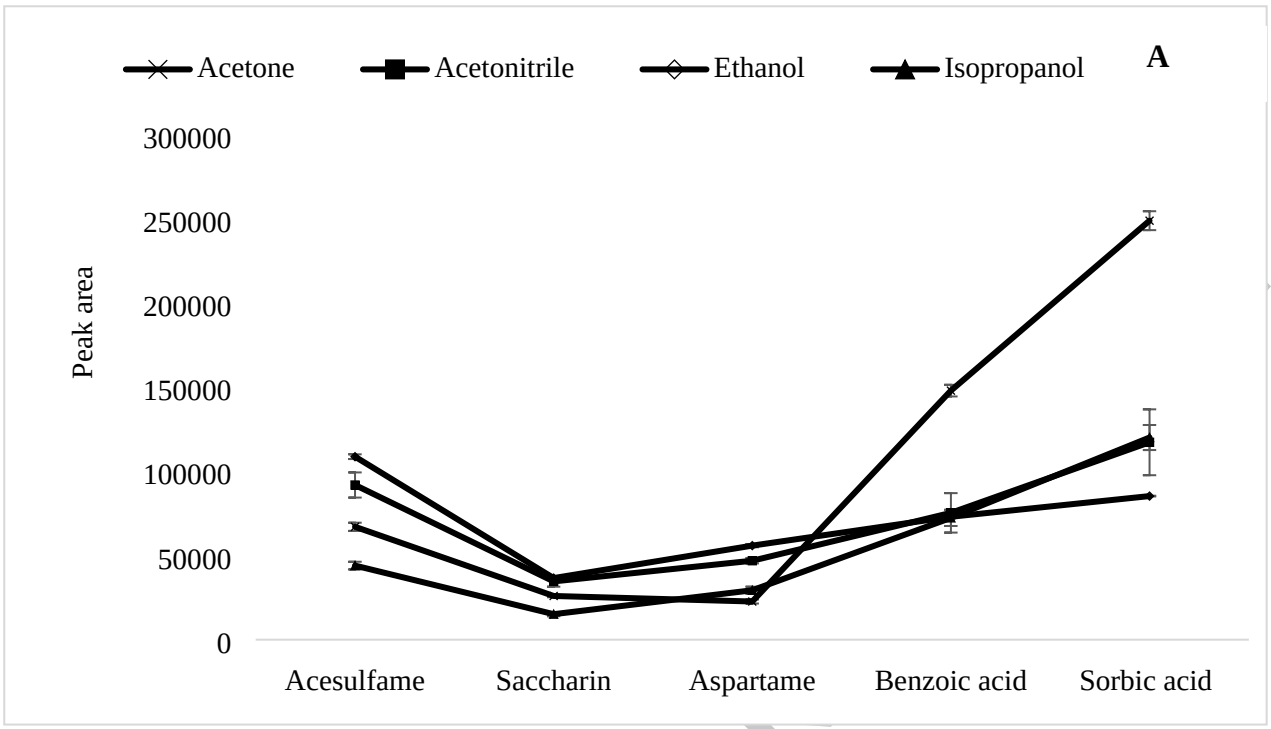

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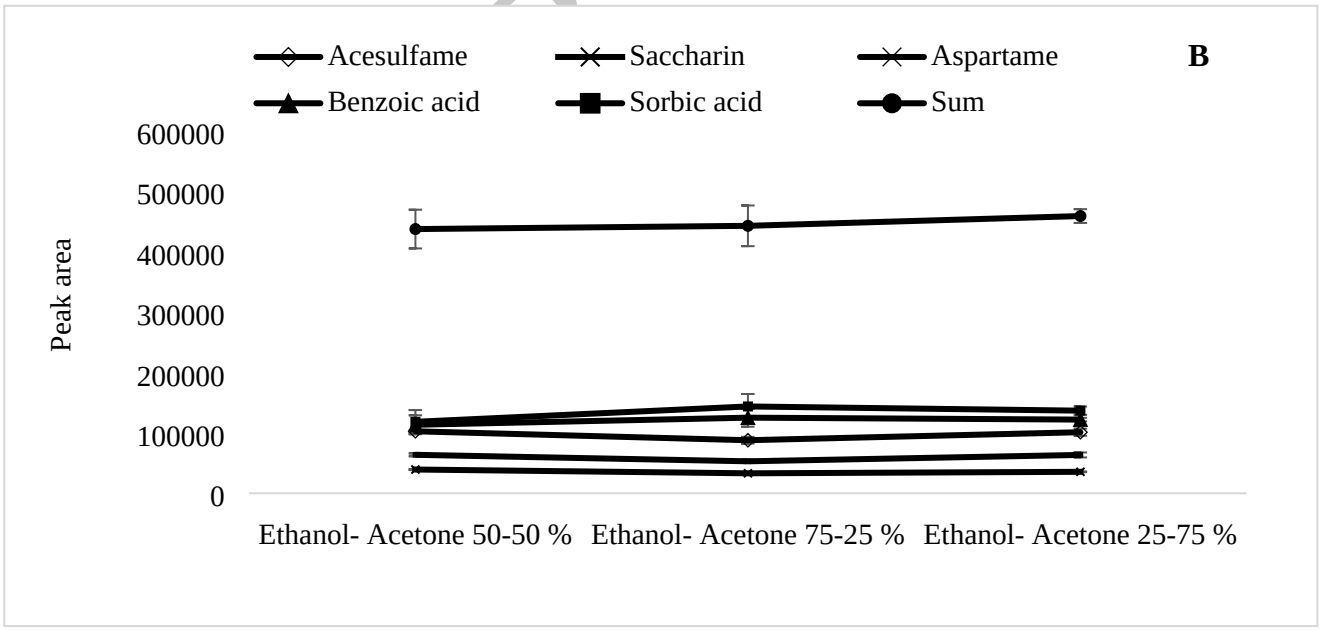

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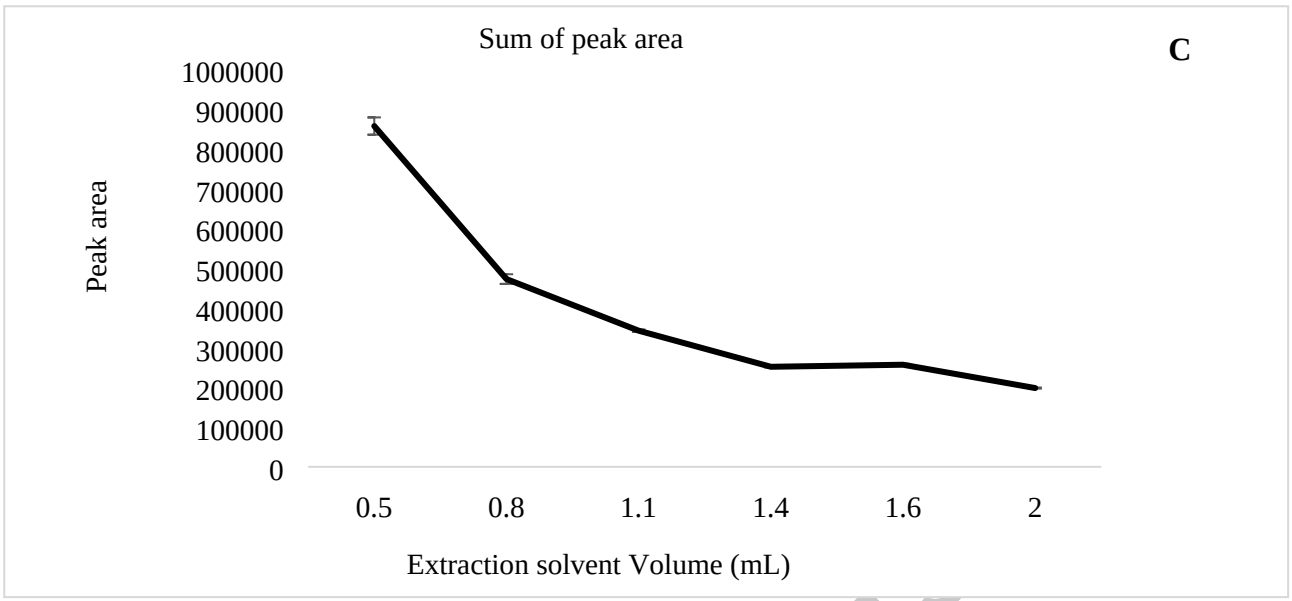


Fig. 1.

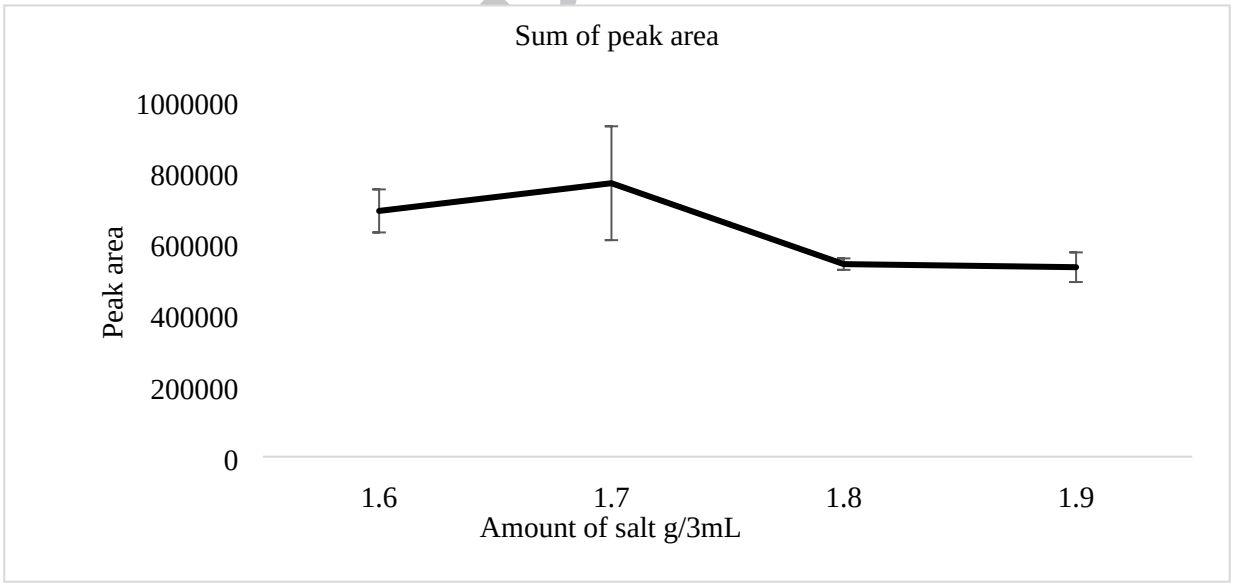
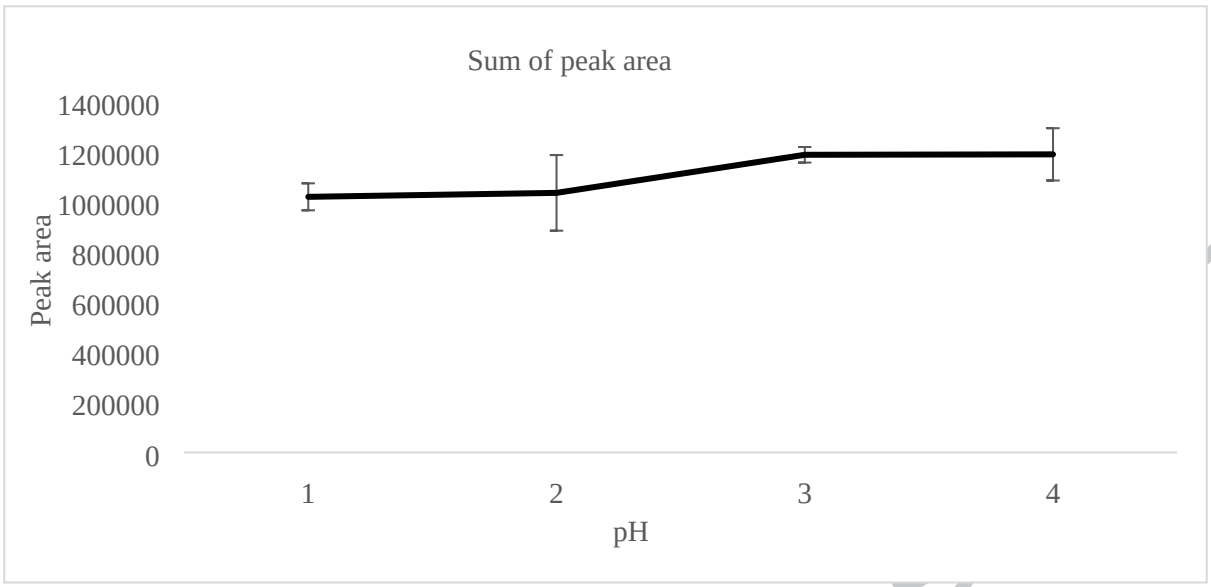


Fig. 2.



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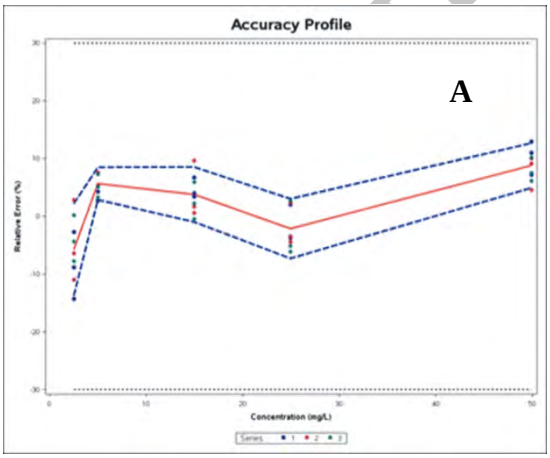

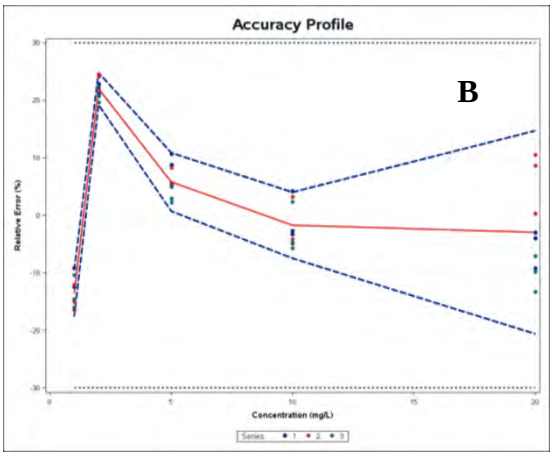

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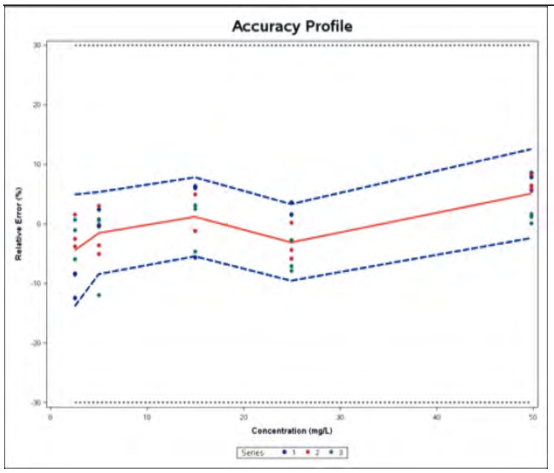
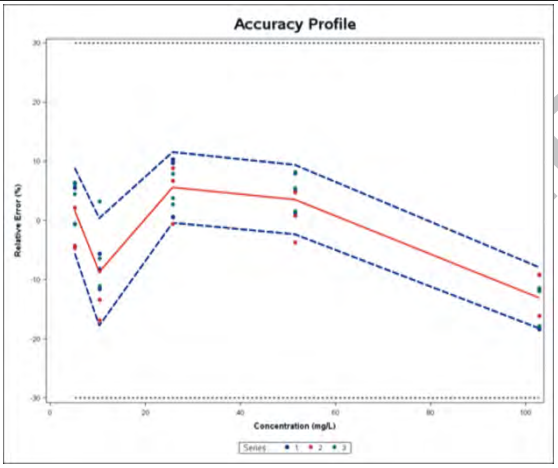
			C				D	
Compounds	Level µg mL^{-1}	Trueness	Precision		LOD	L		R^2
		Recovery %	RSD (R) %	RSD (IP) %	µg mL^{-1}	µg mL^{-1}		





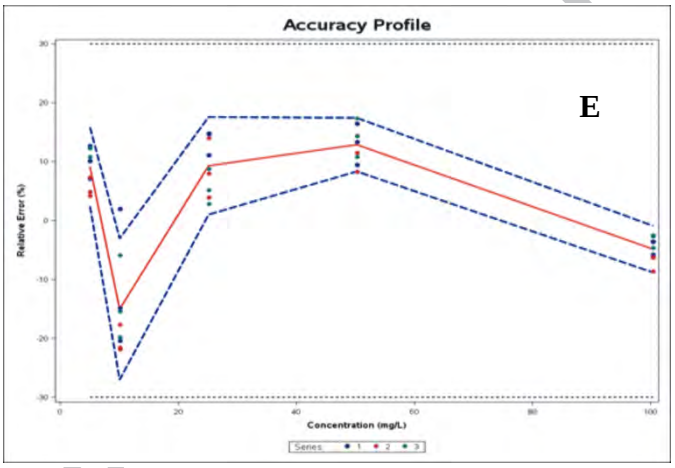


Fig. 4.

Table 1

Acesulfame	2,49	94.22	5.4	5.4	0.75	2.49	0.9958
	4,99	105.7	1.91	1.91			
	14,96	103.8	3.21	3.21			
	24,93	97.87	3.49	3.49			
	49,87	108.8	2.62	2.62			
Saccharin	0.99	86.45	2.72	2.72	0.30	0.99	0.9876
	1.99	122.00	1.27	1.70			
	4.99	105.8	2.33	3.09			
	9.99	98.22	3.89	3.89			
	19.98	97.08	4.10	9.16			
Aspartame	2.49	95.56	2.89	5.15	0.75	2.49	0.9961
	4.98	98.47	4.67	4.67			
	14.94	101.2	4.49	4.49			
	24.89	96.89	3.75	4.19			
	49.79	105.1	1.24	3.68			
Benzoic acid	5.14	101.6	3.74	4.54	1.27	5.14	0.9856
	10.28	91.30	5.16	5.87			
	25.71	105.6	4.05	4.05			
	51.42	103.5	3.67	3.89			
	102.84	86.93	3.49	3.49			
Sorbic acid	5.02	113.6	1.65	4.08	1.42	5.02	0.9876
	10.04	83.20	6.99	7.45			
	25.10	103.0	3.52	4.79			
	50.21	111.3	3.22	3.22			
	100.42	99.06	1.49	2.80			

R, repeatability; IP, intermediate precision

R^2, correlation coefficient for the linearity of back calculated versus introduced concentration

Table 2

		Acesulfame		Saccharin		Aspartame		Benzoic acid	
Brand	n	Label claim	Range µg mL^{-1}	Label claim	Range µg mL^{-1}	Label claim	Range µg mL^{-1}	Label claim	Ran µg
A	6	No	ND	No	ND	No	ND	No	36.34 -7
B	5	No	ND	No	ND	No	ND	No	60.09 -7
C	6	No	ND	No	ND	No	ND	No	0- 102.(
D	5	No	ND	No	ND	No	ND	No	ND
E	6	No	ND	No	ND	No	ND	No	ND
F	5	No	ND	No	ND	No	ND	No	ND
G	5	No	ND	No	ND	No	ND	No	ND
H	4	No	ND	No	ND	No	ND	No	ND
I	4	No	ND	No	ND	No	ND	No	ND

n, number of sample; ND, not detected

[a], standard deviation

Highlights

- Salting out liquid-liquid extraction coupled to HPLC-UV was used for analysis of five food additives
- The method was validated using accuracy profile as decision tool
- Validated method was applied to monitoring 47 commercial samples of fruit juices.
- Linear regression after logarithm transformation used as response function.
- Good recoveries, intra and inter day reproducibility were obtained