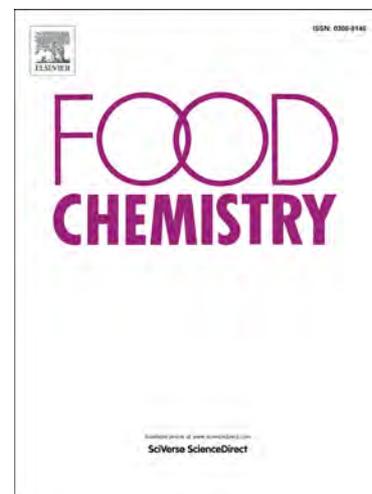# Accepted Manuscript

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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2   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).

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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

72 conjugates of benzoic acid derivatives, mainly as hippuric acid. Sorbic acid is harmless and it is

73 absorbed and mainly excreted or expired as carbon dioxide (WHO, 1974). According to JECFA

74 (WHO, 1993), saccharin is not metabolized and it is suspected to induce bladder cancer in male rat,

75 which is not relevant to human. Pharmacokinetic studies show that acesulfame is completely

76 absorbed, not metabolized and rapidly excreted unchanged and no adverse effects are associated to

77 acesulfame (WHO, 1991). Aspartame is metabolized in the body into aspartic acid, phenylalanine

78 and methanol and high level of phenylalanine in plasma is known to cause developmental toxicity

79 in humans to individuals with inherited metabolic disorder called phenylketonuria (EFSA ANS

80 Panel, 2013). Moreover, as stated by Swithers-Susan (2013), other harmful effects can be associated

81 to artificial sweeteners with negative health outcomes such as risk of weight gain, metabolic

82 syndrome, type 2 diabetes, hypertension and cardiovascular disease. Therefore, the use of

83 preservatives and artificial sweeteners in foodstuffs must be monitored.

84 Recently, several analytical methods were employed for separation and quantification of food

85 additives. High or ultra-high performance liquid chromatography (HPLC or UPLC) coupled to

86 ultraviolet detector (Diogo, Silva, Pena & Lino, 2013; Lino et al., 2010), diode array detector

87 (DAD) (Dias, Meinhart, Pane, Ballus & Godoy, 2015) have been proposed. In addition, tandem

88 mass spectrometry (MSn) (Chang & Yeh, 2014) were the main used techniques. Other techniques

89 were also used and include spectrophotometry UV with PLS-2 method (Cantarelli, Pellerano,

90 Marchevsky & Camina, 2009), capillary electrophoresis (CE) with capacitively coupled contactless

91 conductivity detection (CE-C^{4}D) (Bergamo et al., 2011) and micellar electrokinetic chromatography

92 (MEKC) coupled to UV and DAD (Boyce, 1999;).

93 Sample pretreatment and cleanup is a mandatory step in food samples in order to remove the

94 matrix interference prior to the chromatographic determination. Various sample preparation and

95 simultaneous extraction ~~processes~~ of these five compounds have been reported in literature such as,

96 centrifugation, microfiltration and dilution (Diogo, Silva, Pena & Lino, 2013; Lino et al., 2010).

97 Nevertheless, the importance of these techniques cannot be underestimated, as not all errors that

98 occur in this step can be corrected, even by the best separation or detection method (Szultka,

99 Pomastowski, Railean- Plugaru & Buszewski, 2014). Microextraction method such as dispersive

100 solid-phase extraction (dSPE) using ethylenediamine or tetraethylynepentamine functionalized

101 Fe_3O_4 magnetic polymer (TEPA MP) and (IEPA MP) were developed (Chen, Zhao, Shen & Jin,

102 2012; Zhao, Cai, Chen, Pan, Yao & Jin, 2013). These last extraction techniques are tedious, time

103 consuming and expensive, because of the preparation and the synthesis of the TEPA MP and IEPA

104 MP, which use expensive reagents, and their characterization with transmission electron

105 microscopy, which cannot be available in every laboratory. Moreover, these techniques are used to

106 remove natural pigments, organic acids and sugars from the matrix, where a limiting factor can be

107 the sorption of the analytes on these compounds, and thus, the low recoveries.

108 Non-polar water-immiscible organic solvents used in liquid phase microextraction (LPME) have

109 low dielectric constant and they are relatively poor for extraction of polar compounds. More-polar

110 water-miscible solvents such as acetone, acetonitrile, ethanol and isopropanol, that provide

111 solubility for polar to non-polar compounds, cannot be used for conventional liquid liquid

112 extraction (LLE) or LLME method. However, if the solvent is generated *in situ* in the aqueous

113 solution and a phase separation further occurs, two-phase system is obtained upon the addition of an

114 appropriate quantity of an electrolyte, such as a salt, that decrease the miscibility of two mixed

115 liquids (Tabata, Kumamoto & Nishimoto, 1996). Then, the analytes can move selectively from the

116 aqueous phase into the polar organic phase. This process is called salting out and it is applied in

117 salting out liquid liquid extraction (SALLE) or extraction by demixture (Nerín, Polo, Salafranca &

118 Cacho, 1996). Recently, this technique was applied as a simple, fast, economical, green and benign

119 extraction/cleanup method for the preparation of various samples and extraction of different

120 compounds such as mycotoxins, antibiotics, pesticides, drugs, polyphenolic compounds and metals

121 from different matrices such as water, biological fluids and food (Magiera & Kwietniowska, 2016).

122   The objective of this study is the optimization and validation of fast and efficient salting out

123 liquid liquid extraction (SALLE), coupled to UPLC-UV, for the simultaneous extraction and

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124 determination of food additives with different polarities from the same aliquot of sample. Univariate

125 optimization method was used to optimize the main and effective parameters affecting the

126 extraction, to enhance the efficiency of the method. The optimized method was validated using

127 accuracy profile and applied to the analysis of 47 samples of juices ~~beverage samples~~ to check if

128 these five additives (acesulfame, saccharin, aspartame, benzoic and sorbic acids) were legally used

129 and within the maximum permitted levels.

130   To our knowledge, there are no literature describing the simultaneous extraction and analysis of

131 sweeteners and preservatives with the application of SALLE method. The results based on the

132 application of this method show that the method is simple, exhibits excellent applicability, rapid,

133 cheap, environmentally friendly, and very suitable for extraction of food additives with different

134 polarities. As in SALLE method the extraction solvent is generated in situ, handling of etraction and

135 enrichment factors can be very high, and the complexity of the extraction is considerably reduced.

2. Materials and methods

2.1. Reagents

138   Potassium acesulfame ($\geq$ 99 %), sodium saccharin ($\geq$ 99 %), and aspartame ($\geq$ 99 %), Sodium

139 benzoate ($\geq$ 99 %) and sorbate potassium ($\geq$ 99 %) were obtained from (Sigma Aldrich, Germany).

140 Acetonitrile, acetone, anhydrous ethanol, isopropanol, methanol (HPLC or LC MS grade),

141 hydrochloric acid (HCl, 37%), sodium chloride (NaCl, $\geq$ 99.5) and sodium sulphate (Na$_2$SO$_4$, $\geq$ 99

142 %) were obtained from (Scharlau, Spain). Ultrapure water used for samples preparation through the

143 work was purified from Millipore system (Milli-Q plus 185). Ammonium acetate (CH$_3$COONH$_4$, $\geq$

144 98%), magnesium sulphate heptahydrate (Mg SO$_4$ 7 H$_2$O) and ammonium chloride (NH$_4$Cl, $\geq$ 99.8

145 %) were obtained from (Merck, Germany). Ammonium sulphate ((NH4)$_2$ SO$_4$, 99%) was purchased

146 from (Pancreac Appli Chem Barcelona, Spain) and Trifluoroacetic acid (TFA, 99 %) was purchased

147 from (Fluka, Switzerland). Flow of N$_2$ (ALPHAGAZI, 99.999% Global purity, Air Liquide

148 Zaragoza-Spain) was used for the evaporation of extraction solvent.

6

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

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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).

3. Results and discussion

198 *3.1. Optimization of the method*

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199   Fruit juices are complex matrices containing sugars, and fibers ~~flavonols and preservatives~~

200   among other compounds at low concentration such as proteins. For this reason, sample pretreatment

201   and cleanup is a mandatory step in order to isolate the compounds of interest. In this work, different

202   compounds with different polarities as amino and carboxylic groups were the target. Hence, the

203   simultaneous extraction of these compounds with water-immiscible organic solvents is not an easy

204   task. The low dielectric constants of these solvents and the polar nature of these compounds, which

205   are slightly or very slightly soluble in immiscible water solvent, make the extraction of the

206   mentioned compounds quite poor, mainly for saccharin, aspartame and acesulfame. However, all

207   these compounds are soluble in water miscible solvents with high dielectric constant i.e. ethanol,

208   acetone, acetonitrile and isopropanol. Hence, SALLE using these water miscible solvents was

209   selected for simultaneous extraction of these compounds. These solvents can dissolve in water in

210   any proportion because of "hydrogen bond" interaction. The presence and the increasing amount of

211   ammonium sulphate in aqueous solution, containing ethanol and acetone, leads to the migration of

212   water molecules away from ethanol and acetone molecules to ions of salts and the mass fraction of

213   acetone and ethanol increases in the upper phase (Wang, Yan, Hu, Han & Xu, 2010). Therefore, at

214   saturated concentration of the ammonium sulphate two phases are formed *in situ*.

215   For this purpose, 3 mL of ultrapure water spiked with standards at concentration of 20 µg mL^{-1}

216   for acesulfame, aspartame, benzoic and sorbic acids and at 4 µg mL^{-1} for saccharin was used for

217   method optimization using univariate optimization method, by varying one factor while keeping all

218   the other factors at constant level. The main factors affecting the extraction efficiency measured as

219   the chromatographic peak area were the nature and the volume of the extraction solvent, the amount

220   of salt and the pH of sample. All the results are the average of triplicate measurements.

221   Optimization of factors influencing the extraction method is discussed below in detail and the

222   optimized results were 0.5 mL for the mixture of ethanol: acetone at (50: 50 v/v) as extraction

223   solvent, 1.7 g/ 3mL of ammonium sulphate used as salting out agent and pH 3.

224   *3.1.1. Selection of extraction solvent type and volume*

225   Selection of extraction solvent is an important consideration and crucial step to control the

226   efficiency of SALLE process. This solvent must be polar, miscible with water in all proportions,

227   with high capability to dissolve the analytes and easily separable from water by adding an

228   electrolyte as salt. The saturated concentration of the salts in the presence of different solvents is

229   different for each compound. Therefore, different tests were performed to determine the saturated

230   concentration of ammonium sulphate in water with achieving a salting out effect in the presence of

231   the tested extraction solvents i.e. (acetone, ethanol, acetonitrile and 2-propanol). These amounts

232   were found and ranged from 1.7 to 2.05 g. In order to select the most appropriate organic solvent, 1

233   mL of each solvent was tested in the mixture of 3 mL of sample and different ammonium sulphate

234   amounts. The results are shown in fig. 1A. As can be seen, the maximum responses were obtained

235   with ethanol for acesulfame, saccharin and aspartame and with acetone for benzoic and sorbic acids.

236   Therefore, the mixtures of both ethanol and acetone at different ratios of (50: 50; 75: 25 and 25: 75

237   v/v) were tested. As shown in fig. 1B, the sum of peak areas was identical. However, the ratio (50:

238   50, v/v) gave the best results for saccharin used at very small concentration in juices products.

239   Therefore, the binary mixture of ethanol: acetone at (50: 50 v/v) was used as suitable extraction

240   solvent.

241   The volume of extraction solvent was studied at 0.5, 0.8, 1.1, 1.4, 1.6 and 2 mL. The results

242   illustrated in fig. 1C show that as the volume of extraction solvent increases the sum of peak areas

243   for all the compounds decreases and the results are higher for 0.5 mL of extraction solvent. This is

244   because when increasing the volume of extraction solvent, the amount of analytes transferred

245   increases too and more food additives are extracted from the aqueous phase. However, the dilution

246   effect reduces the analyte concentration with high extraction solvent volume. Therefore, 0.5 mL

247   was selected as the optimum volume for the extraction.

248   *3.1.2. Selection the type and the amount of salt*

249   As it is known the salting out for phase separation between organic extraction solvent and the

250   aqueous solution varies with the type of salt added. Therefore, different organic and inorganic

251   salting out agents other than ammonium sulphate were checked at their saturated concentration of

252   1.08, 3.7, 1.42 and 1.15 g for sodium chloride, magnesium sulphate heptahydrate, sodium sulphate

253   and ammonium chloride, respectively. The results showed that the best two-phase separation was

254   obtained for ammonium sulphate. For sodium and ammonium chloride, two phases were not

255   obtained. This is probably due to the presence of methanol in the sample used for the preparation of

256   aspartame. Moreover, these salts have low salting out effect because these salts are with monovalent

257   anion and cation (Lu, Hao, Hu, Han, Tan & Yan, 2013). For magnesium sulphate heptahydrate, the

258   two phases were not clearly separated. In the case of sodium sulphate, an important salting out

259   effect was observed mixture of extraction solvent and water was released. This is probably due to

260   the transference of the aqueous phase into the organic solvent. Therefore, ammonium sulphate that

261   gave the best two-phases separation was selected for the rest of the work.

262   The ionic strength also influences the solubility of the analytes in the sample solution. Therefore,

263   the effect of the concentration of salt on the extraction efficiency was studied with addition of

264   different ammonium sulphate amount, ranging from 1.6 to 1.9 g/ 3 mL. As shows fig. 2, when the

265   concentration of salt increases from 1.6 to 1.7 g, the response for the sum of peak areas increases

266   too. This can be explained by the hydration theory, as when the concentration of salt ions in water

267   increases, more water molecules form preferentially hydration sphere around the salt ions, and

268   fewer water molecules are available to hydrate the organic compounds (Kokosa, Przyjazny &

269   Jeannot, 2009). This is explained by the difference in the acting force between an "ion-water" and

270   an "alcohol-water" pair, leading to separation of organic phase rich in analytes (Wang et al., 2010).

271   However, the response for the sum of peak areas decreases when the amount of salt is added at high

272   concentrations of 1.8 and 1.9 g. This can be explained by the increase of viscosity of the aqueous

273   solution, which result in a difficult mass transfer.

274 *3.1.3. Selection* the *pH of sample*

275   The pH of the sample is a significant factor affecting the extraction efficiency and the transfer of

276   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.

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303 Based on the accuracy profile results, several acceptable response functions were obtained for each

304 compound. However, linear regression after logarithm transformation was selected as the simplest

305 one and applied as regression model for all the studied compounds.

306 The precision of the method evaluated at each level of VSs provides information regarding the

307 random error describing the degree of which data generated from replicate measurements differ

308 from one to another. It was evaluated at two levels of repeatability (intra assay precision) and

309 intermediate precision (inter day precision) by measuring the relative standard deviation. The

310 results are shown in Table 1. The RSD % values obtained were very good and they ranged from

311 1.24 to 7.99 % for repeatability and from 1.69 to 9.16 % for intermediate precision.

312 The trueness of the method measured at each concentration level express the difference between

313 the obtained analytical value and the true value accepted as reference. It can be estimated in

314 absolute or relative bias or in term of absolute or relative recovery. In solvent extraction method,

315 relative recovery defined as the ratio of the back-calculated concentrations found in the investigated

316 matrix to those in distillated or deionized water is generally used. The results show that the relative

317 bias is good and ranged between 1.19 and 22.05 %. Moreover, the obtained recoveries are

318 satisfactory and ranged from 84.97 and 122 % for all the compounds.

319 The linearity of the method was evaluated and determined by fitted linear regression curve

320 obtained by plotting back calculated concentrations of validation standards versus theoretical or

321 introduced concentration and applying the best selected model for each compound. All the

322 regression fitted well with minimal coefficients R^2 value ≥ 0.99, showing the good linearity of the

323 results generated by the SALLE-UPLC-UV method.

324 Accuracy express the closeness of agreement between the test results and the accepted reference

325 or conventionally true value. The total error is taken into account, which is the sum of trueness

326 (systematic error) represented by the relative bias (%) and precision (random errors) represented by

327 the lower tolerance limit (LTL) and the upper tolerance limit (UTL). The accuracy profiles given in

328 fig. 4 show that 80% of the β expectation tolerances limits for all compounds are inside the

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329   acceptance limits set at ± 30 %. This means that the analytical SALLE-UPLC-UV method is

330   capable of making accurate results over the whole concentration range for all the compounds.

331   Consequently, the lower and upper quantitation limits (LLOQ and ULOQ) are the extreme

332   values that can be quantified with a defined accuracy and were [2.49-49.87], [0.99-19.98], [2.49-

333   49.79], [5.14, 102.84], [5.02, 100.42] for acesulfame, saccharin, aspartame, benzoic acid and sorbic

334   acid, respectively. As the accuracy profile approach is used for method validation, the LOQ

335   correspond to the LLOQ that can be detected and quantified accurately. The results summarized in

336   table 1 show that the LOQs ranged from 0.99 to 5.02 µg mL^{-1}.

337   The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that

338   can be detected but not necessarily quantified as an exact value. In this work, the LOD was

339   calculated using the methodology described by Miller & Miller (1993). This method is based on the

340   computation by series of the Y_{LOD} = Intercept (0 if negative) + 3 x Residual SD obtained by

341   ANOVA. Using the selected regression model, the back-calculation will give the X_{LOD} for each

342   series. The mean of the different back-calculated X_{LOD} will give the LOD of the procedure. The

343   results summarized in table 1 show that the LODs ranged from 0.3 to 1.42 µg mL^{-1}.

344 *3.3.   Analysis of real samples and occurrence of additives*

345   The developed method was successfully applied for the determination and quantification of three

346   artificial sweeteners and two preservatives in 47 juices products belonging to nine different brands

347   from Algerian market. The results from all analyzed samples are summarized in table 2. As regards

348   to acesulfame, saccharin and aspartame, fortunately, none of these compounds were found in any

349   analyzed product and these results are in accordance with the Taiwan study of Chang et al. (2014).

350   However, all the other studies found and quantified at least one sweetener in the analyzed samples

351   of juices (Bergamo et al., 2011; Dias et al., 2015; Diogo et al., 2013; Lino et al., 2010).

352   Preservation technology such as pasteurization, aseptic packaging and the high acidic character

353   (pH 3- 4) of fruit juice can be used for protection of fruit juices against the growth of

354   microorganisms (Cakir & Cagri-Mehmetoglu, 2013). It is important to highlight that benzoic and

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355 sorbic acids or their salts are forbidden in juices and nectars by Algerian food additive legislation

356 (Ministère du commerce Algérien, décret exécutif n°12-214. 2012). However, potassium sorbate

357 was found declared in the label claim of two brands of analyzed samples but their concentrations

358 were not given.

359   The results of the analyzed samples show that both benzoic and sorbic acids were detected and

360 quantified separately in 12 samples (25.53%) and simultaneously in 6 samples (12.76%). The mean

361 concentrations measured were 63.03 ± 14.23 and 65.69 ± 21.76-µg mL^{-1} ranging from 36.34 to

362 102.09 and 32.34 to 118-µg mL^{-1} for benzoic and sorbic acids or their salts, respectively and a

363 maximum of 171.6 µg mL^{-1} for the sum of the two compounds when detected simultaneously. The

364 results were not in accordance with the guidelines of Algerian standard legislation (Ministère du

365 commerce Algérien, Décret exécutif n°12-214. 2012) as the use of these additives in juices is

366 forbidden ; however, they were below or did not surpassed the allowed limit of 1000 µg mL^{-1} set by

367 Codex Alimentarius (2016).

368 The results found in this work are lower for benzoic acid and very similar for sorbic acid to those

369 found by the Portuguese study of Mota et al. (2003). Moreover, as in our study, the researchers did

370 not detect exceeding levels of benzoic or sorbic acids in fruit juices.

371   The study of Cakir & Cagri-Mehmetoglu (2013) showed that only one juice sample contained

372 benzoic acid at 181.4 µg mL^{-1}. Moreover, benzoic and sorbic acids were not detected in fruit juices,

373 and only traditional soft drinks or soft drinks based on mineral water had those additives (Diogo et

374 al., 2013). The results found were not too high to be dangerous for the consumers. The

375 concentration levels found could be from the indigenous and natural content of fruits (Davidson, et

376 al., 2001) or resulted from the contamination of fruits used in the preparation of the samples of

377 juices by air, rain, soils and water which could contain benzoic acid (Javanmardi et al., 2015).

378 **4. Conclusion**

379   In this study, a simple, fast, economical, green and benign extraction/cleanup method namely

380 SALLE coupled to UPLC-UV was applied for routine monitoring and quantitative determination of

15

381 five food additives with wide range of physicochemical properties namely acesulfame, saccharin,

382 aspartame, benzoic and sorbic acids in samples of juices. This method reduces the consumption of

383 the extraction solvent and sample volumes to a very low level compared to the classic extraction

384 methods such as LLE and SPE. The method was optimized using univariate methodology and

385 validated using accuracy profile approach based on the total error. Under the optimum extraction

386 conditions, the optimized method provides suitable trueness, precision, extraction recoveries,

387 linearity, accuracy and sensitivity in a short time. The method was successfully applied to the

388 analysis of samples of juices to ensure a good quality of products and compliance with additive

389 legislation, in order to avoid the effects of these sweeteners and preservatives on people's health if

390 used at high concentrations. The results show that only benzoic and sorbic acids were detected and

391 quantified in the analyzed samples and there are no problems regarding the concentrations found

392 which were not of concern for consumer health.

393

394 **Funding**

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527 Figure captions

528 Fig. 1. Influence of (A) Extraction solvent type, (B) Ratio of acetone and ethanol and (C) Volume

529 of extraction solvent on SALLE efficiency. Extraction conditions: (A) extraction solvent volume, 1

530 mL; ammonium sulphate amount, 1.7-2.05 g, 1.7-2.05 g / 3mL and pH, 2; (B) extraction solvent

531 volume, 1 mL ; salt concentration, 1.7 g/ 3 mL and pH, 2; (C); Extraction solvent, ethanol: acetone

532 (50, 50 v/v); salt concentration, 1.7 g/ 3 mL and pH, 2.

533 Fig. 2. Influence of salt amount for SALLE efficiency. Extraction conditions: Extraction solvent,

534 ethanol: acetone (50, 50 v/v); Solvent volume, 0.5 mL and pH, 2.

535 Fig. 3. Influence of pH on SALLE efficiency. Extraction conditions: Extraction solvent, ethanol:

536 acetone (50, 50 v/v); Solvent volume, 0.5 mL; salt amount, 1.7 g/ 3 mL.

537 Fig. 4. Schematic representation of the accuracy profiles obtained for all the compounds. (A)

538 Acesulfame; (B) Saccharin; (C) Aspartame; (D) Benzoic acid and (E) Sorbic acid. The dotted lines

539 are the lower and upper acceptance limits set at 30 %, the dashed lines are the lower and upper β-

540 expectation tolerance limits and the continuous line is the relative bias.

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547 Tables

548 Table 1: Results of analytical performances of SALLSE- UPLC-UV method for the quantification

549 of the five food additives in juice sample (p= 3, m= 5 and n= 3).

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

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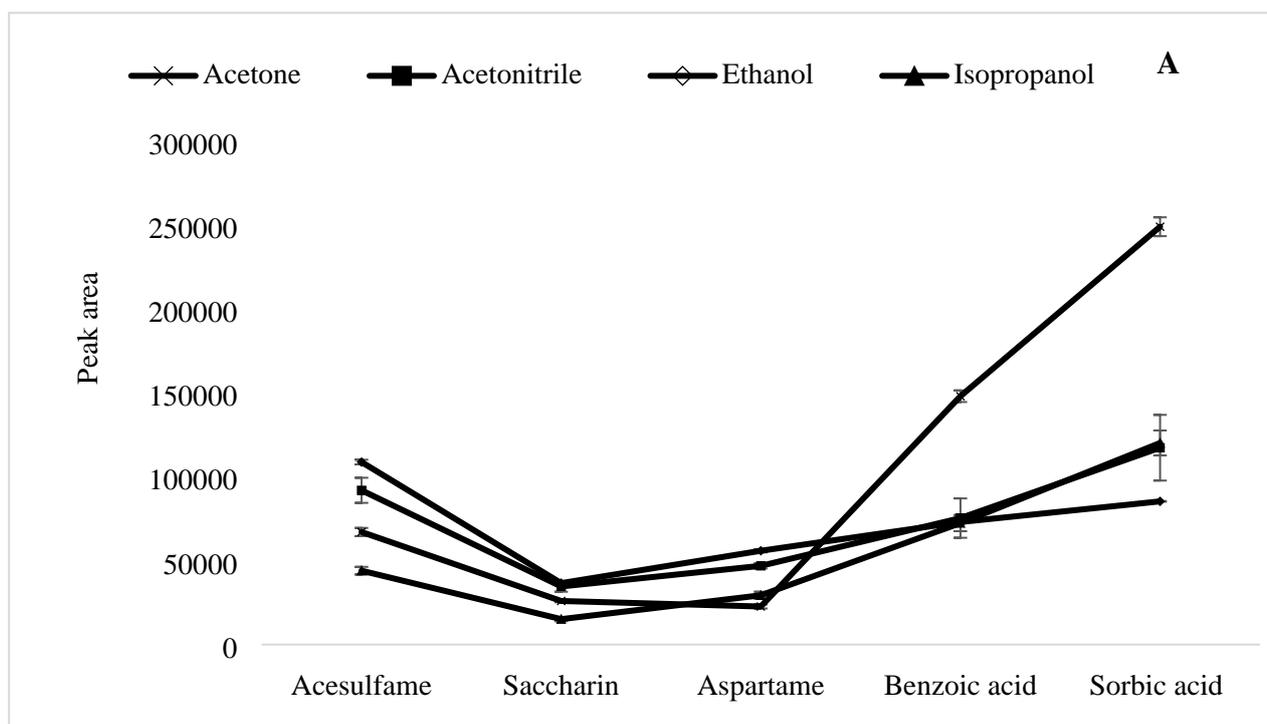
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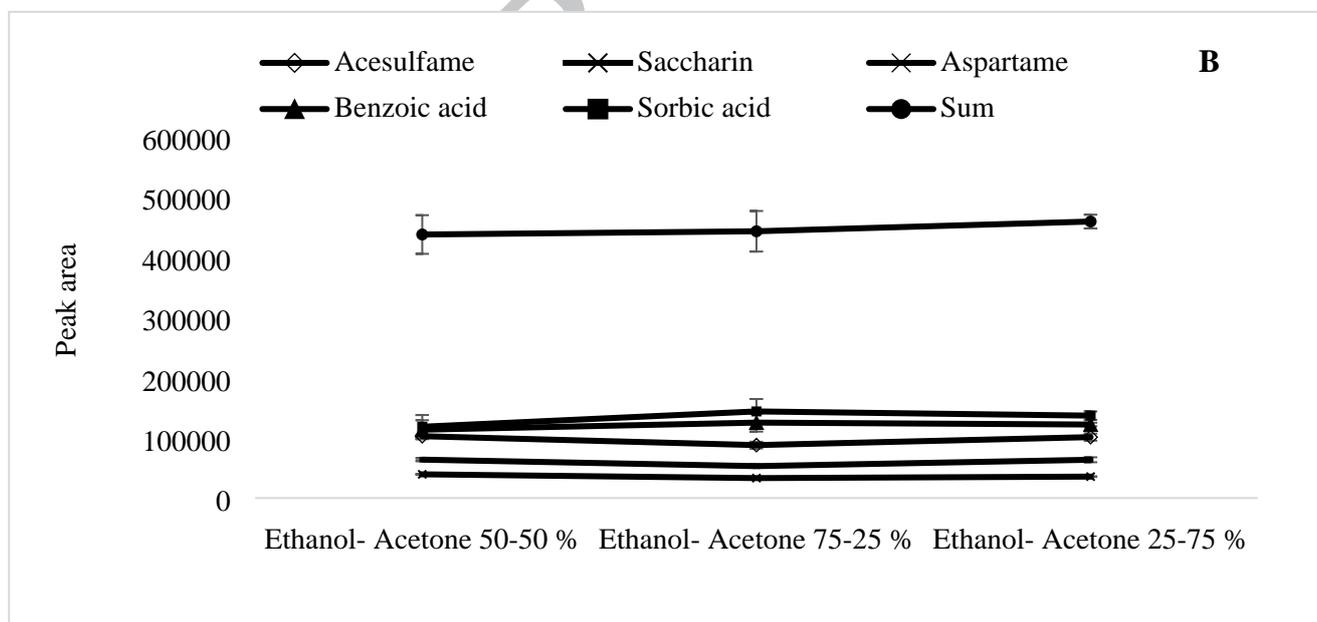
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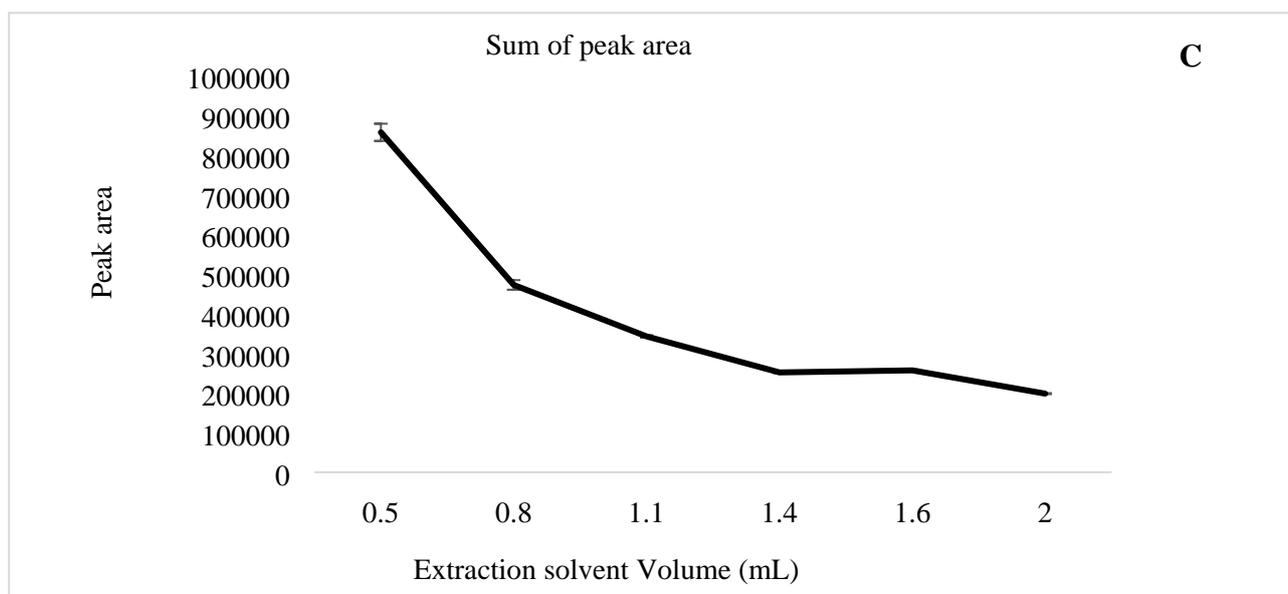
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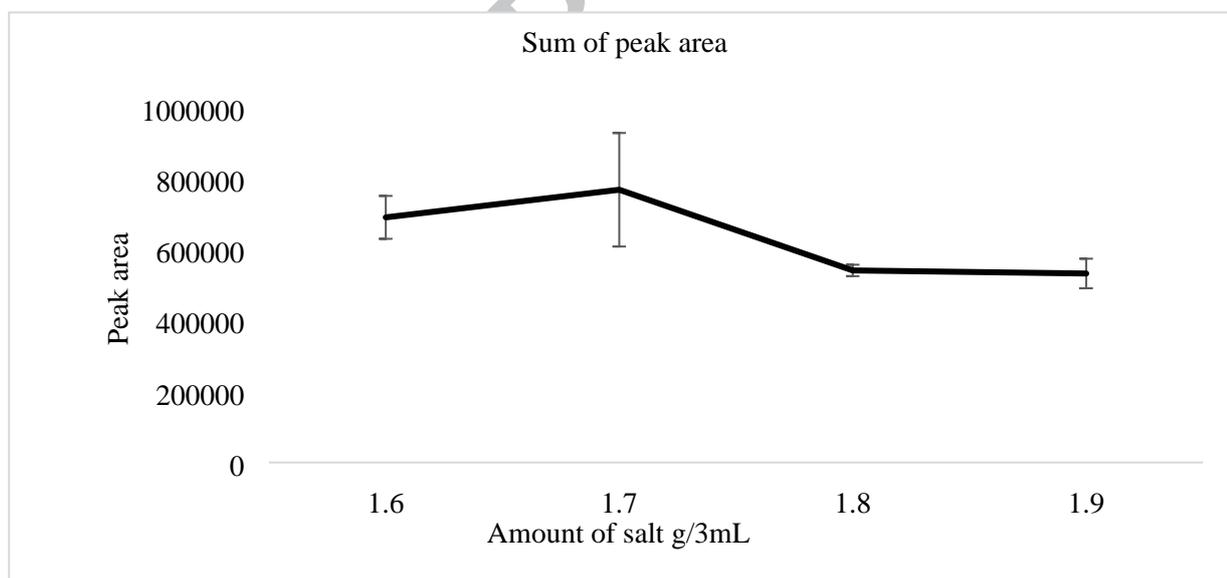
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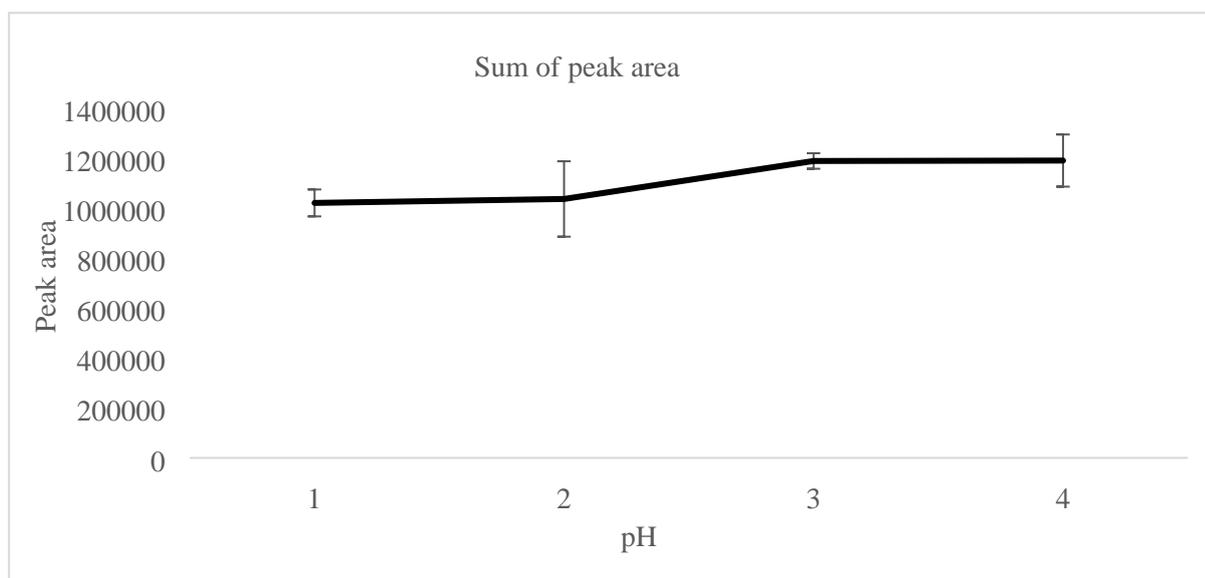
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577   Fig. 1.

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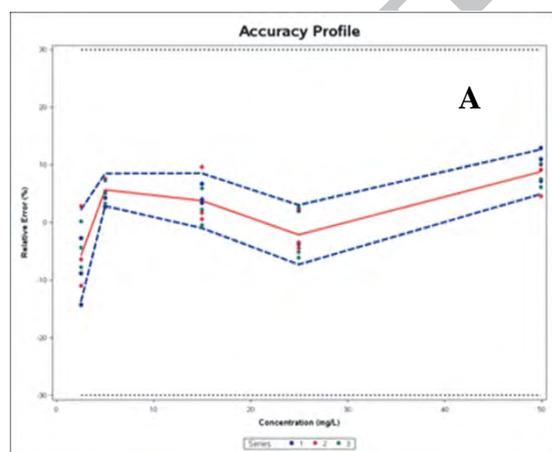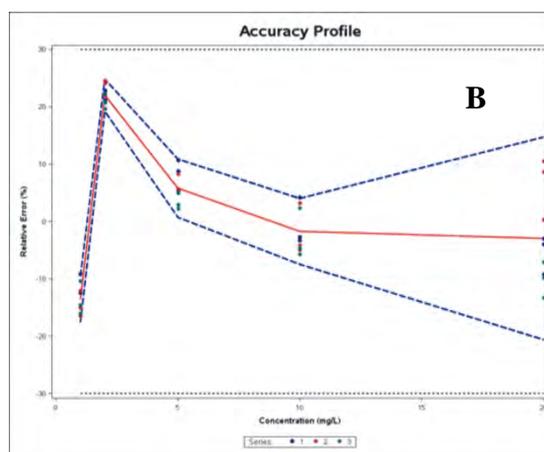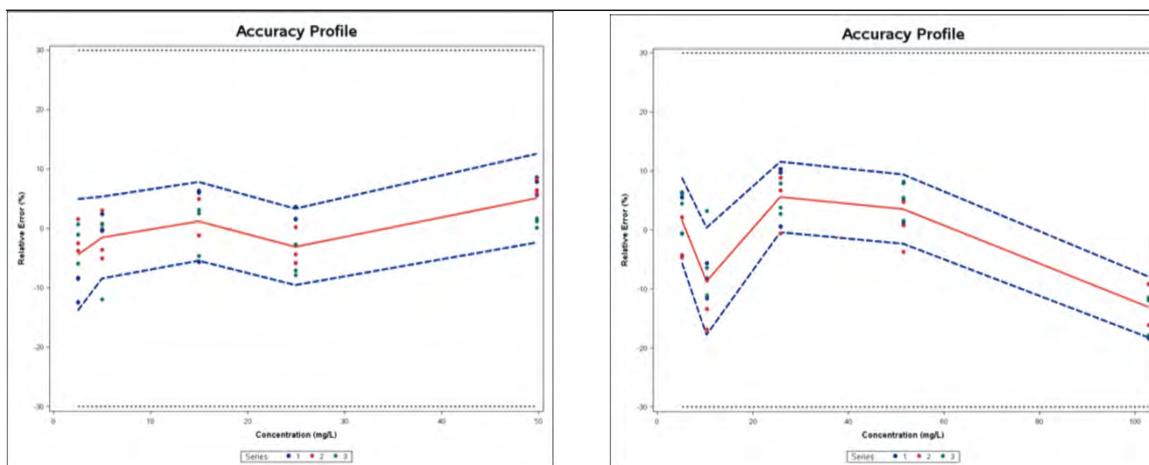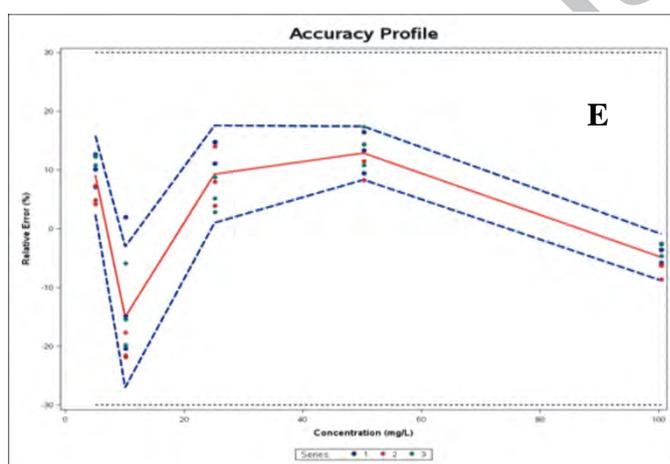

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Compounds	Level µg mL⁻¹	C Trueness Recovery %	Precision		LOD µg mL⁻¹	D	R²
			RSD (R) %	RSD (IP) %		µg mL⁻¹	

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595 Fig. 4.

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603 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			

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606   R, repeatability; IP, intermediate precision

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

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611   Table 2

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		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	Range µg ᵢ
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- 102.0
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

613

614 n, number of sample; ND, not detected

615 [a], standard deviation

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618 Highlights

619 • Salting out liquid-liquid extraction coupled to HPLC-UV was used for analysis of five food

620 additives

621 • The method was validated using accuracy profile as decision tool

622 • Validated method was applied to monitoring 47 commercial samples of fruit juices.

623 • Linear regression after logarithm transformation used as response function.

624 • Good recoveries, intra and inter day reproducibility were obtained

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