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Application of Quality-by-Design Approach in the Analytical Method Development for Quantification of Sugars in Sugarcane Honey by Reversed-Phase Liquid Chromatography

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Abstract

Produced in Madeira Island from regional sugarcane cultivars through a traditional manufacturing and storage process, sugarcane honey (SCH) is a black syrup recognized by its excellent quality. Its economic value has led to the emergence of adulterated SCH, whereby the identification of molecular markers became an essential task in order to overcome the fraudulent activities, protect its authenticity, and guarantee the consumer safety. In the present study, an analytical strategy based on ultrasound-assisted liquid-liquid extraction (USA-LLE) followed by reversed-phase liquid chromatography with a refractive index detector (LC-RI) was developed for the determination of sugars (glucose, fructose, sucrose, xylose, and mannose) in SCH samples from certified producers, supported on analytical quality-by-design (AQbD) approach, as a useful tool to establish its typicality. The application of AQbD was based on analytical risk assessment, multivariate statistics and quality control procedures for definition of the Method Operable Design Region (MODR). The optimal conditions into MODR were accomplished using BEH Amide column operating at a temperature of 80 °C and a flow rate of 300 μL min^{-1}, with a mobile phase composed by acetone and water (85:15, $v\ v^{-1}$) at a flow rate of 1.0 mL min^{-1}. The robustness was determined by Monte Carlo simulation and capability analysis. The concentration-response function for all sugars was described by polynomial models. Accuracy was presented by recovery values between 98.2 and 119.5%. The analytical figures of merit validated the utility of AQbD in the systematic design of a LC-RI method with fine sensitivity for sugar analysis in SCH.

Keywords Sugarcane honey · Sugar · Typicality · Quality-by-design · USA-LLE · LC-RI

Introduction

Food authenticity has become a global problem, increasing the importance of the establishment of typicality markers to guarantee the authenticity of products and consumer safety. The sugarcane honey (SCH), known as "*mel-de-cana*," is a black syrup produced in Madeira Island, Portugal, commonly used as a main ingredient in regional pastry and confectionery, being also consumed fresh and in traditional sauces for meat, fish and salads (Silva et al. 2017a; Silva et al. 2018). The manufacturing process of SCH was developed for more than a century, which started in late years of XIX century, since then, it has become a part of the historical and cultural patrimony of the region. This manufacturing process is based only in thermal treatment and filtering procedures of juice obtained by mechanical pressing of fresh stalks from regional sugarcane (*Saccharum officinarum* L.) cultivars, originating the worldwide recognized quality and unique organoleptic properties of SCH (Silva et al. 2017b). However, recently some low-quality products have been introduced in the market labeled as SCH that not respect the traditional manufacturing process or the use of regional cultivars. Many of these low-quality products are obtained as by-products from the sugar refining, alcoholic beverages and biofuel production industries, where refined sugars, corn syrups, invert syrups, or other

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low-cost syrups are also often added to reduce production costs (Silva et al. 2017a; Silva et al. 2018). These cases of product adulteration are threatening significantly the notoriety of SCH. Thus, it is imperative to develop strategies for establishment of typicality of SCH to guarantee its authenticity.

Sugars are the most abundant compounds found in nature, being widely used as molecular markers in food analysis domain for determination of geographical origin (Nikolaou et al. 2017; Coelho et al. 2018; Karabagias 2019), typicity (Ghfar et al. 2015; Mellado-Mojica et al. 2016; Kek et al. 2017) and authenticity (Cordella et al. 2005; Willems and Low 2012; Wang et al. 2015) of several food products. The sugar profile influences strongly the nutritional, sensory, and organoleptic properties of the final product, such as sweetness, viscosity, crystallization, granulation, and energy value (Özbalci et al. 2013; Georgelis et al. 2018). Moreover, several important active aroma and taste compounds are formed by thermal degradation (e.g., Maillard reactions, Strecker degradation, caramelization) and microbial activity (e.g., bacterial, yeast activity) on sugars during manufacturing and storage processes (Silva et al. 2017b; Georgelis et al. 2018). Although sucrose, glucose, and fructose are normally the main components of thermal processed sugary-products, such as agave syrup (Willems and Low 2012; Muñiz-Márquez et al. 2015), yacon syrup (de FG da Silva et al. 2018), date syrup (Al Eid 2006), maple syrup (Mellado-Mojica et al. 2016), raspberry syrup (Grembecka et al. 2014), beet molasse (Vaccari et al. 2001), cane honey (Seguí et al. 2015), sugarcane molasse (Xu et al. 2015), and inverted sugar syrup (Cordella et al. 2005), its profile varies depending on raw materials, manufacturing process, and storage. In this context, the establishment of sugars profile of SCH from certified producers can be a valuable strategy to define its typicality.

Recently, a wide variety of analytical methods have been proposed for sugars analysis of food products, synthesized in Table 1. Although some alternative analytical methods have been developed for the sugars analysis, such as gas chromatography (GC) coupled to mass spectrometry (MS) (Rodríguez-Sánchez et al. 2011; Guadalupe et al. 2012) or flame ionization detector (FID) (Willems and Low 2012; Idda et al. 2016), nuclear magnetic resonance (NMR) (Jamróz et al. 2014b), high performance thin layer chromatography (HPTLC) (Vaccari et al. 2001; Terol et al. 2010), capillary zone electrophoresis (CZE) (Rovio et al. 2011; Wang et al. 2012) and Raman spectroscopy (Özbalci et al. 2013). High performance anion exchange liquid chromatography with pulsed amperometry detection (HPAEC-PAD) (Cordella et al. 2005; Anjos et al. 2015; Seguí et al. 2015; Mellado-Mojica et al. 2016) and high performance liquid chromatography (HPLC) combined with refractive index detection (RI) (Al Eid 2006; Xu et al. 2015; Muñiz-Márquez et al. 2015; de FG da Silva et al. 2018) or evaporating light scattering detection (ELSD) (Ma et al. 2014; Kek et al. 2017; Lindqvist et al. 2018) are the most widely applied methods for sugar profile of food products. Other types of detection are also used in combination with HPLC, namely mass spectrometry (MS) (Ghfar et al. 2015; Georgelis et al. 2018), fluorescence (FLD) (Rakete and Glomb 2013), ultraviolet (UV) (Bai et al. 2015), and charged aerosol detection (CAD) (Grembecka et al. 2014). However, FLD and UV detections require a time-consuming derivatization step before analysis, while MS and CAD detectors are too expensive for routine analysis (Ni et al. 2016; Koh et al. 2018). Although the HPAEC has been successfully applied to sugar profiling of some food products, the high-pH eluents that are required can generate interferents by epimerization or degradation of food components. In addition, poor resolution of sucrose is normally obtained in HPAEC-PAD analysis (Rakete and Glomb 2013; Koh et al. 2018). On the other hand, HPLC-ELSD and HPLC-IR are two popular methods for determination of underivatized sugars analysis without the use of high-pH eluents. However, HPLC-ELSD requires high amount of expensive nebulizer gas and presents low reproducibility and sensitivity in analysis of low molecular weight sugars (Ma et al. 2014; Magwaza and Opara 2015). Alternatively, HPLC-IR is a simple, fast and economical method that does not require additional gas or complex eluent gradient, being successfully used to sugar profiling of various food products, such as milk (Chávez-servín et al. 2004), juice (Nikolaou et al. 2017), wine (Coelho et al. 2018), fruits (Cantín et al. 2009; Filip et al. 2016), honey (Wang et al. 2015; Karabagias 2019), syrups (Al Eid 2006; Muñiz-Márquez et al. 2015; de FG da Silva et al. 2018), molasses (Xu et al. 2015), among others.

The lack of harmonization and quality processes in development of these analytical methods for sugar profiling hampers its application as a routine and precise tool in determination of typicity of food products. For example, during the development of most methods described in Table 1, an optimization of the parameters that affect its performance is not performed, and when performed, is based on the univariate strategy. Likewise, usually an appropriate method validation procedure is not applicated. Thus, the applicability of analytical information obtained by these described methods in other food products is complicated, being imperative the implementation of quality and harmonized procedures in development of analytical methods.

Analytical quality-by-design (AQbD) is an effective and successful approach for quality assurance in the development of an analytical method based on risk assessment, multivariate statistics, and control quality procedures, being recognized by European Medicines Agency (EMA) and Food and Drug Administration (FDA) regulators as a performance qualifier of analytical methods (EMA and FDA 2013; Silva et al. 2017b). The International Conference on Harmonization (ICH) in Guideline ICHQ8(R2) (ICH 2009) define AQbD as "a systematic approach to development that begins with

Table 1 Analytical methods for determination of sugars in food matrices

Sugars	Food product	Study propose	Sample preparation	Analysis	Optimization type	Validation parameters	Reference
Fructose, glucose, galactose, sucrose, lactulose, lactose	Milk	Sugar profile	LLE	LC-RI	n.p.[a]	Linearity, accuracy, precision, LOD, LOQ	(Chávez-Servín et al. 2004)
Fructose, glucose; sucrose, sorbitol	Peach, nectarine	Cultivars	LLE	LC-RI	n.p.[a]	n.p.[a]	(Cantín et al. 2009)
Fructose, glucose; sucrose, sorbitol	Apple	Cultivars	UA-LLE	LC-RI	Box-Behnken design (H2SO4 concentration, flow rate, column temperature)	Linearity, accuracy, precision, LOD, LOQ	(Filip et al. 2016)
Fructose, glucose	Orange juice	Geographical origin	n.p. [a]	LC-RI	n.p.[a]	Linearity, accuracy, precision, LOD, LOQ	(Nikolaou et al. 2017)
Maltose, glucose, fructose, rhamnose	Grape juice, wine	Geographical origin	n.p. [a]	LC-RI	n.p. [a]	Linearity, accuracy, precision, LOD, LOQ	(Coelho et al. 2018)
Fructose, glucose, sucrose, maltose	Honey	Geographical origin	LLE	LC-RI	n.p. [a]	Linearity, precision, LOD, LOQ	(Karabagias 2019)
Fructose, glucose, sucrose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose	Honey	Adulteration	n.p. [a]	LC-RI	n.p. [a]	n.p.[1]	(Wang et al. 2015)
Fructose, glucose, sucrose	Sugarcane molasses	Sugar profile	SPE Clean-up	LC-RI	Univariate design (column type, mobile phase)	Linearity, accuracy, precision, LOD	(Xu et al. 2015)
Fructose, glucose; sucrose; kestose	Agave syrup	Sugar profile	n.p. [a]	LC-RI	n.p. [a]	n.p. [a]	(Muñiz-Márquez et al. 2015)
Fructose, glucose, sucrose, maltose	Yacon syrup	Sugar profile	LLME	LC-RI	n.p. [a]	n.p. [a]	(de FG da Silva et al. 2018)
Fructose, glucose, sucrose	Date syrup	Sugar profile	n.p. [a]	LC-RI	n.p. [a]	n.p. [a]	(Al Eid 2006)
Fructose, glucose, sucrose	Nectar	Sugar profile	n.p. [a]	LC-ELSD	n.p. [a]	Linearity, Accuracy, Precision, LOD, LOQ	(Lindqvist et al. 2018)
Fructose, glucose; sucrose, sorbitol	Peach, apple, watermelon, cherry fruits	Sugar profile	n.p. [a]	LC-ELSD	Univariate design (column temperature, mobile phase, gas flow rate)	Linearity, accuracy, precision, LOD, LOQ	(Ma et al. 2014)
Fructose, glucose, sucrose	Honey	Typicality	n.p. [a]	LC-ELSD	n.p. [a]	n.p. [a]	(Kek et al. 2017)
Fructose, glucose, sucrose, maltose, lactose, erythritol, xylitol, sorbitol, mannitol,	Candies, gum, jelly, chocolate	Sugar profile	LLE	UPLC-ELSD	Univariate design (column type, mobile phase)	Linearity, accuracy, LOD, LOQ	(Koh et al. 2018)

Table 1 (continued)

Sugars	Food product	Study propose	Sample preparation	Analysis	Optimization type	Validation parameters	Reference
inositol, maltitol, lactitol, isomalt							
Fructose, glucose; sucrose, maltose, maltotriose	Honey, inverted sugar syrup	Adulteration	n.p. [a]	HPAEC-PAD	n.p. [a]	n.p. [a]	(Cordella et al. 2005)
Trehalose, glucose, fructose, sucrose, melezitose, turanose, maltose	Honey	Sugar profile	n.p. [a]	HPAEC-PAD	n.p. [a]	LOQ	(Anjos et al. 2015)
Fructose, glucose, sucrose	Cane honey, jaggeries, brown sugar	Sugar profile	n.p. [a]	HPAEC-PAD	n.p. [a]	n.p. [a]	(Seguí et al. 2015)
Fructose, glucose, sucrose	Maple syrup	Typicality	n.p. [a]	HPAEC-PAD	n.p. [a]	n.p. [a]	(Mellado-Mojica et al. 2016)
Rhamnose, fructose, glucose,sucrose, lactose, raffinose, maltose, erlose	Honeydew, nectar	Sugar profile	UA-LLE	HPAEC-PAD	Univariate design (column type, mobile phase, sample elimination)	Linearity, LOD, LOQ	(Ni et al. 2016)
Fructose, glucose, sucrose	Potato, strawberry	Typicality	LLE	LC-MS	Univariate design (extraction method)	Selectivity, linearity, accuracy, precision, LOD, LOQ	(Georgelis et al. 2018)
Fructose, glucose,sucrose, kestose, nystose	Palm fruit dates	Sugar profile	LLE	UPLC-MS	Univariate design (column type, mobile phase)	Linearity, accuracy, precision, LOD, LOQ	(Ghfar et al. 2015)
Glucose, fructose, sucrose, maltose, erythritol, mannitol, maltitol, sorbitol, xylitol	Orange-apple juices, black currant-cherry nectars, raspberry syrup	Sugar profile	n.p. [a]	LC-CAD	Univariate design (column temperature, mobile phase, column flow rate)	Linearity, accuracy, precision, LOD, LOQ	(Grembecka et al. 2014)
Ribose, xylose, glucose, maltose, isomaltose, maltotriose, maltopentaose, maltohexaose, maltoheptaose	Beer	Sugar profile	Derivatization	LC-FLD, LC-MS	n.p. [a]	n.p. [a]	(Rakete and Glomb 2013)
Isomaltose, isomaltotriose, panose, maltose, glucose	Wine rice	Sugar profile	Derivatization	LC-UV	n.p. [a]	Linearity, accuracy, precision, LOD, LOQ	(Bai et al. 2015)
Maltoheptose, maltohexose, maltopentose, maltotetrose maltotriose, maltose, sucrose, glucose, fructose, xylose, rhamnose	Beet molasses	Sugar profile	Derivatization	HPTLC-FLD	n.p. [a]	n.p. [a]	(Vaccari et al. 2001)
Ribose, glucose, fructose, galactose, inositol, galactinol	Mulberry	Sugar profile	LLE, derivatization	GC-MS	Univariate design (derivatization procedure)	Linearity, accuracy, precision, LOD, LOQ	(Rodríguez-Sánchez et al. 2011)
Apiose, arabinose, rhamnose, fucose, xylose,	Wine	Sugar profile	Derivatization	GC-MS, GC-FID	n.p. [a]	Linearity, accuracy,	(Guadalupe et al. 2012)

Table 1 (continued)

Sugars	Food product	Study propose	Sample preparation	Analysis	Optimization type	Validation parameters	Reference
mannose, galactose, glucose						precision, LOD, LOQ	
Glucose, lactose, galactose, inositol	Milk	Sugar profile	Derivatization	GC-FID	Univariate design (injection volume)	Linearity, accuracy, precision, LOD, LOQ	(Idda et al. 2016)
Fructose, glucose, sucrose, mannitol, inositol	Agave syrup	Authenticity	n.p. [a]	GC-FID, HPAEC-P-AD	n.p. [a]	n.p. [a]	(Willems and Low 2012)
Fructose, glucose, sucrose, maltose	Honey	Sugar profile	n.p. [a]	Raman spectroscopy	n.p. [a]	n.p. [a]	(Özbalci et al. 2013)
Fructose, glucose, sucrose, maltose	Honey	Sugar profile	n.p. [a]	NMR	n.p. [a]	n.p. [a]	(Jamróz et al. 2014a)
Maltose, lactose, xylose, arabinose, glucose, ribose, rhamnose, fucose, galactose, mannose	Beer, milk	Sugar profile	LLE, Derivatization	CZE-UV	n.p. [a]	Linearity, accuracy, precision, LOD, LOQ	(Wang et al. 2012)
Cellobiose, fructose, fucose, galactose, glucose, inositol, mannitol, mannose, rhamnose, ribose, sorbitol, trehalose, xylose	Wine	Geographical origin	n.p. [a]	CZE-UV	n.p. [a]	Linearity, accuracy, precision, LOD, LOQ	(Rovio et al. 2011)

LOD limit of detection, *LOQ* limit of quantification, *LLE* liquid-liquid extraction, *CZE-UV* capillary zone electrophoresis-ultraviolet, *NMR* nuclear magnetic resonance, *GC-FID* gas chromatography-flame ionization detection, *HPAEC-PAD* high performance anion exchange liquid chromatography with pulsed amperometry detection, *GC-MS* gas chromatography-mass spectrometry, *HPTLC-ICP-AES* high performance thin layer chromatography, *HPTLC-FLD* high performance thin layer chromatography-fluorescence detector, *HPTLC-UV* high performance thin layer chromatography-ultraviolet, *HPLC-MS* high performance liquid chromatography-mass spectrometry, *HPLC-CAD* high performance liquid chromatography-charged aerosol detection, *UPLC-MS* ultra performance liquid chromatography-mass spectrometry, *LC-ELSD* - high performance liquid chromatography-evaporating light scattering detection, *UPLC-ELSD* - ultra performance liquid chromatography-evaporating light scattering detection, *LC-RI* - high performance liquid chromatography-refractive index detection, *USA-LLE* ultrasound-assisted liquid-liquid extraction, *SPE* solid phase extraction

[a] Not performed in study

predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." Normally, the workflow of AQbD for development of an analytical method is based on the follow key stages: (i) selection of analytical target profile (ATP), where the analytes, extraction technique, and analysis equipment are selected according the purpose of study; (ii) definition of critical method attributes (CMAs), which are representative of the analytical performance of method, such as peak area, peak resolution, peak asymmetry and retention time; (iii) method scouting through preliminary studies, where are evaluated which parameters have the potential to influence the CMAs; (iv) selection of parameters by quality risk assessment (QRA) based on the Ishikawa diagram according scouting phase results; (v) determination of critical method parameters (CMPs), where the parameters that significantly affect the analytical performance of method are defined; (vi) definition of knowledge space (KS) by design of experiments screening to explore the effects of the CMPs on the CMAs; (vii) definition of method operable design region (MODR) through statistical analysis of method responses based on the interactions between the CMPs and CMAs, such as Pareto analysis, response surface methodology and desirability analysis; (viii) evaluation of method robustness by its capacity to remain unaffected by small deliberate variations on the MODR; (ix) method control by establishment of system suitability limits on the MODR; (x) validation of method by determination of selectivity, calibration function, linearity, precision, accuracy, matrix effect, limits of detection (LOD) and quantitation (LOQ); (xi) applicability of method to real samples (Borman et al. 2007; Hanna-Brown et al. 2010; Molnár et al. 2010; Silva et al. 2017b; Ancillotti et al. 2018). Although the AQbD approach has been widely applied in the pharmaceutical industry, only in last years has started to be introduced

in food analysis. The first application of AQbD approach in development of an analytical method for food analysis was described in our previously study, where AQbD procedures was applied for identification and quantification of furan derivatives in authenticity of SCH samples (Silva et al. 2017b; Silva et al. 2018). Recently, other two studies were published, where the analytical methods were developed based on AQbD framework for determination of polyphenols in diospyros fruit (Ancillotti et al. 2018) and cereals (Balli et al. 2020). The application of AQbD principles is a promising strategy to improve the confidence, control and harmonization of analytical methods and facilitate the exchange of analytical information in food analysis.

The objective of the present study was to develop a robust and precise strategy based on ultrasound-assisted liquid-liquid extraction (USA-LLE) combined with reversed phase LC-RI, employing AQbD approach, for quantification of sugars (glucose, fructose, sucrose, xylose, and mannose) in SCH samples from a certified producer during three consecutive production years (2016, 2017 and 2018), as a powerful strategy to define its typicality.

Materials and Methods

Samples, Standards, Reagents, and Materials

SCH samples were provided by the certified producer Fábrica de Mel-de Cana do Ribeiro Sêco in April 2016 (FRS16), 2017 (FRS17), and 2018 (FRS18) (Madeira Island, Portugal), and stored under stable conditions (4 °C, in the dark). Sugars standards, glucose (GLU), fructose (FRU), sucrose (SUC), xylose (XYL), mannose (MAN), and rhamnose (RHA) were purchased from Sigma-Aldrich (St. Louis, USA). Acetone (ACT), acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), and triethylamine (TEA) were purchased from Sigma-Aldrich (St. Louis, USA). Ultrapure deionized water (H_2O), purified with a Milli-Q ultra-pure water system from Millipore (Massachusetts, USA). All solvents and samples were filtered through 0.22 μm membrane filters from Millipore (Massachusetts, USA), before analysis.

Sugar Standard Solution Preparation

Sugar standard stock solutions (100 g L^{-1}) were prepared by 500 mg of each standard in 5 mL H_2O:EtOH (50:50%, $v\ v^{-1}$), and stored at − 20 °C. Under these conditions, the standards solutions are stable for at least 3 months. Working solutions of lower concentrations with all sugars standards were daily prepared by appropriate dilution with H_2O:ACT (50:50%, $v\ v^{-1}$).

USA-LLE

USA-LLE procedure was performed adding 2.5 g SCH and 7.5 mL H_2O:ACT (50:50%, $v\ v^{-1}$) into a 50 mL PTFE centrifuge tube, followed by homogenization (MAXI MIX Vortex Mixer, from Thermo Scientific, MA, USA) for 2 min and ultrasonication (BRANSON 2510 ultrasonic cleaner, from Bransonic, CT, USA) for 10 min. After this, the extraction solution was centrifuged (ROTOFIX 32A, from Hettich, Kirchlengern, Germany) at 4000 rpm for 15 min. A 2-mL aliquot from the upper part of the extraction solution (ACT phase) was transferred into an 8-mL glass flask. Before injection into the HPLC-RI system, all extracts were filtered through 0.22-μm membrane filters and transferred to HPLC vials.

LC-RI Conditions

The sugar analysis was performed on a HPLC ULTIMATE 3000 series system acquired from Dionex, CA, USA, equipped with a binary pump, an autosampler and a column compartment, coupled to a refractive index detector SHODEX R1-101 from Thermo Scientific, Massachusetts, USA. The instrument configuration and analysis were achieved with Chromeleon™ Chromatography Data System Software from Thermo Scientific, Massachusetts, USA. The chromatographic separation of sugars was carried on a X-Bridge BEH Amide XP (2.5 μm, 4.6 × 100 mm) column acquired from Waters Corporation, MA, USA. The binary mobile phase was composed by H_2O with 0.05% TEA (eluent A) and ACT (eluent B), with an isocratic gradient (15% A and 85% B) at a constant flow rate at 300 μL min^{-1}. The temperatures were strictly controlled, and the column was kept at 80 °C, sample manager at 20 °C and IR flow cell at 50 °C. The injection volume was set at eight μL. The sugars identification was based on the retention times (RTs) of sugar standards, being quantified by the standard calibration curves method.

Statistical Software

All data analysis and statistical processing were performed using the STATSOFT STATISTICA 12.0 (2013) software (Tulsa, USA).

Results and Discussion

The analytical method was developed according the AQbD approach, based on the procedure described in our previous study (Silva et al. 2017b), adapted from recommendations defined in ICHQ8(R2) (ICH 2009) guideline. The workflow chart for AQbD approach is described in Online Resource 1 (Supplementary Material).

ATP, Critical Quality Attributes, and Method Scouting

The ATP of this study was defined by separation, identification, and quantification of GLU, FRU, SUC, XYL, and MAN in SCH samples from a certified producer during three consecutive production years (2016, 2017, and 2018) through USA-LLE/LC-RI analytical method. FRU, GLU, and SUC were selected in this study because these are the most abundant sugars in sugarcane-based food products. In addition, these sugars are also present in widely used adulterants, being added individually or as ingredient of corn syrups, beet syrup, invert syrups, or other low-cost syrups (Başar and Özdemir 2018). Similarly, MAN (Hu et al. 2016) and XYL (Saska and Ozer 1995) were also selected for their potential use as alternative sugary adulterants.

The definition of CMAs was based on chromatography performance of the analytical method, where were selected the total peak area (TPA), peak resolution (PR), and peak asymmetry (PA) of each sugar standard, as well the respective relative standard deviation (RSD). The limits for CMAs values were established according the minimal requirements for a satisfactory chromatography performance, where was mandatory the identification of each sugar standard based on TPA and RT, PR values greater than 1.5, PA values lower than 1.5, and RSD values not higher than 15% (Thompson et al. 2002).

The method scouting was performed by several preliminary experiments. The extraction performance of analytical method was evaluated by comparison between LLE and USA-LLE, and other extraction-influencing parameters (data not shown). The results (TPA and RSD) obtained by USA-LLE were considerable superior than LLE. The use of ultrasound in the LLE possibly provides a better dispersion of the target sugars from samples to the extraction phase (ACT). However, no differences were observed between the different tested times (10, 20, and 30 min) of ultrasound. In addition, three different extraction solvents were evaluated, ACT, can, and MeOH, being that only the extraction performed with ACT provided satisfactory results. The poor results obtained by ACN and MeOH may be due to the fact that the mobile phase used in the chromatographic analysis contains high content (85%) of ACT. Also, different extraction solvent contents were studied (50, 60, and 70%) and no differences were observed. Similarly, the ratios 1:1, 1:2, and 1:3 ($w\ v^{-1}$) between sample and extraction solution (H_2O and ACT) were evaluated. The ratio 1:3 was selected to avoid problems related with high viscosity of SCH samples. The scouting of chromatography performance was based on results from WATERS technology brief (Benvenuti and Burgess 2012), where the analysis of FRU, GLU, and SUC in several fruit juices was performed by LC-RI with a X-Bridge BEH Amide XP column and a binary mobile phase (15% H_2O with 0.05% TEA and 85% ACT) in isocratic gradient mode.

QRA and CMPs

The selection of CMPs was performed by QRA through an Ishikawa diagram constructed according the results obtained on method scouting step, being displayed in Online Resource 2. Although several extraction-influencing parameters have been studied, no CMPs were selected for USA-LLE. On previous scouting step, none of studied parameters demonstrated that its range variability can affect the extraction efficiency of the method. The previously investigated parameters for USA-LLE were kept during further steps of method development. On the other hand, the chromatography performance can be affected by variability of several parameters from LC-RI analysis. The selected CMPs were the type of eluent B, content (%) of eluent B, column flow rate, and column temperature.

Knowledge Space

The WATERS technology brief (Benvenuti and Burgess 2012) provide an excellent source of valuable information for definition of influencing CMPs ranges and screening of KS. A full factorial design (FFD) based on 4^3-level factors model was applied as design of experiments for KS screening, being investigated the eluent B (ACT, ACN, and MeOH), eluent B content (75, 80, and 85%), column flow rate (300, 400, and 500 μL min^{-1}), and column temperature (60, 70, and 80 °C). Unexpectedly, the mobile phases with ACN and MeOH did not allow the separation of sugars under analysis. Moreover, ACN and MeOH caused a co-elution of all compounds, making it impossible to identify the five sugars under analysis. Although the most studies published of sugar analysis based on LC-RI use only aqueous eluents (Wang et al. 2015; Coelho et al. 2018), organic eluents, such as ACN, already have been used successfully. For example, GLU, FRU, and SUC were separated and identified in molasses samples, utilizing an Utimate XB-NH2 column (5 μm, 4.6 × 250 mm) with a binary mobile phase composed by ACN and H_2O (75:25%, $v\ v^{-1}$) (Xu et al. 2015). Other study also used a binary mobile phase composed by ACN and H_2O (40:60%, $v\ v^{-1}$) in a Zorbax RX-SIL column (5 μm, 4.6 × 250 mm) for analysis of GLU, FRU, and SUC in honey samples (Karabagias 2019). Possibly, in our study, the combination of the short length (100 mm) and amide groups of column promotes a rapid elution of sugars, not allowing them to separate with these two organic eluents, even using a low column flow rate (300 μL min^{-1}). For this reason, the FFD for KS screening was really based on 3^3-level factors model. The CMAs (TPA, PR and PA) results, CMPs and respective range values of KS FFD screening model are summarized in Online Resource 3 to Online Resource 5, respectively.

Method Operable Design Region (MODR)

The definition of MODR was based on LC analysis, wherein the CMA responses from the interactions of previous selected CMPs were evaluated through the 3^3-level factors model FFD defined previously in KS. The optimum CMAs responses from interactions between CMPs were exhaustively explored through Pareto ranking analysis (PRA), response surface methodologies (RSM), and desirability analysis (DA) in order to define an MODR where the analytical method will achieve the purposed ATP. The MODR definition was achieved through the TPA, PR, and PA responses obtained from ACT content, column flow rate, and column temperature, and from its interactions. The PRA for TPA, PR, and PA are shown in Fig. 1 a, b, and c, respectively.

According to results, all CMPs demonstrated, individually, a significant influence on the TPA response, being that its influence decreased in the following order: column flow rate > ACT content > column temperature. However, only the interaction between ACT content and column flow rate presented a significant influence on the TPA response. Likewise, all CMPs showed an individual and significant influence on the PR response, wherein its influence decreased according this order: ACT content > column temperature > column flow rate. The PR response was also significantly influenced by interaction between the ACT content with column flow rate and column temperature. Regarding the results from PA response, the ACT content and column temperature were the only CMPs with a significant effect. Thus, all CMPs confirmed its significant influence on CMAs responses, and consequently, in definition of MODR.

The RSM plots of interaction between the range level of continuous CMPs (ACT content vs column flow rate, ACT content vs column temperature, and column flow rate vs column temperature), on TPA, PR, and PA responses are presented in Fig. 2a–c, d–f, and g–i, respectively.

Based on results, it was verified that the higher TPA values were obtained by the content of 85% ACT with a column flow rate of 300 μL min^{-1} and a column temperature of 80 °C. The ACT content below 85% and column flow rate above 300 μL min^{-1} caused a clearly decrease in TPA values. On the other hand, only smooth differences were observed between the studied ranges of column temperature, where 80 °C showed the high TPA values. The best values for PR and PA were also obtained with 85% ACT, 300 μL min^{-1} and 80 °C, being that range values below 85% ACT and 80 °C demonstrated a large drop in PR values and an increase in PA values. The range values of column flow rate only showed slight differences on the PR and PA response.

The DA was performed by overlay of CMAs responses to establish the optimal range values of CMPs and obtain the highest desirability index, and consequently, define the MODR. The desirability values for TPA, PR, and PA were based on the optimum, medium and poor response results, being defined as: desirable (392, 2.2, and 1.0), acceptable (237, 1.8, and 1.1), and unacceptable (127, 1.4, and 1.2), respectively. The desirability analysis plots for interactions between CMPs, namely the ACT content vs column flow rate, ACT content vs column temperature, and column flow rate vs column temperature, are presented in Fig. 3 a, b, and c, respectively.

As expected, the highest desirability index was obtained in a region close to optimum conditions point, where the CMPs values were 85% of ACT, 300 μL min^{-1} of column flow rate and 80 °C of column temperature, being that the region of interaction around the optimum point was defined as the MODR of analytical method. The data verification of MODR was based on the analysis of agreement between the predicted and observed values at optimum point, being described in Online Resource 6. The results from data verification analysis demonstrated small differences between the observed and predicted values in CMA responses. However, the value of difference between the observed and predicted values was always close to 1%. The observed differences may be due to the fact that the predicted values were calculated by the response of CMAs based only in three levels of CMPs that cover a wide range. Consequently, slight variations on intermediate precision at the optimum point can promote a visible

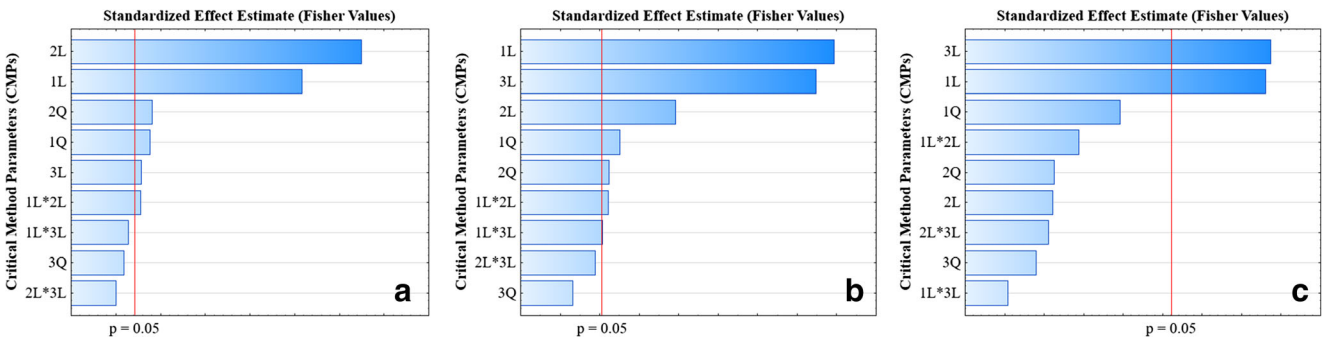

Fig. 1 Pareto chart plot of standardized effects of CMPs for definition of MODR regarding the total peak area (**a**), peak resolution (**b**), and peak asymmetry (**c**) results. Abbreviations correspond to (L) linear function model and (Q) quadratic function model. Numbers correspond to (1) ACT content, (2) column flow, and (3) column temperature

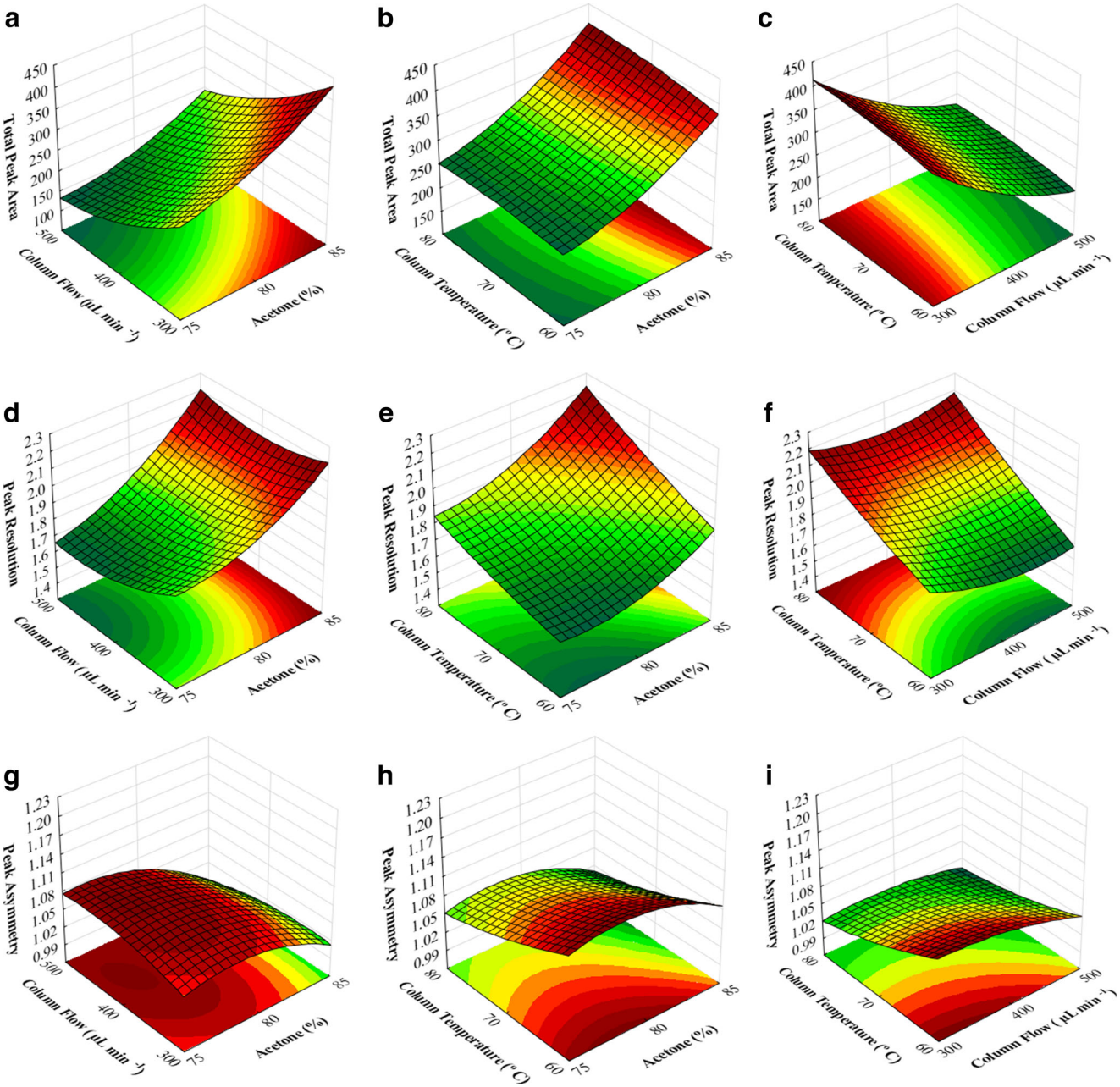

Fig. 2 Response surface methodology plot of interactions between the continuous CMPs, ACT content vs column flow, ACT content vs column temperature, and column flow vs column temperature, for definition of MODR regarding the total peak area (**a**, **b**, and **c**), peak resolution (**d**, **e**, and **f**), and peak asymmetry (**g**, **h**, and **i**) results, respectively

variability in the CMAs responses. For this reason, the MODR was evaluated by the analysis of the robustness at CMPs levels more closed to the optimal point, providing a rigorous and strict control of analytical method.

Robustness and Method Control

The control strategy of an analytical method performance during routine applications was based on evaluation of robustness, being normally achieve through the introduction of small and deliberate variations on the optimum conditions to verify if the analytical performance to remain unaffected. The evaluation of robustness was based on a new design of experiments performed at the optimum point into MODR. A fractional factorial design based on 3^3-level factors model was performed for robustness screening, where were evaluated the ACT content (84, 85, and 86%), column flow (290, 300, and 310 μL min^{-1}), and column temperature (79, 80, and 81 °C).

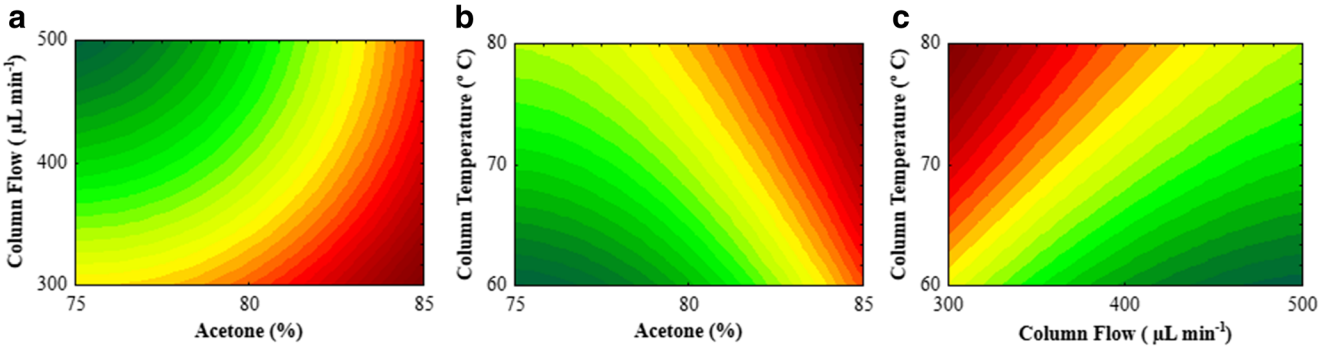

Fig. 3 Desirability analysis index plot of interactions between the continuous CMPs, ACT content vs column flow (**a**), ACT content vs column temperature (**b**), and column flow vs column temperature (**c**), for definition of MODR

The TPA, PR, and PA responses, CMPs, and respective range values for robustness screening model are summarized in Online Resource 7, 8 and 9, respectively.

The DA was performed on results obtained from robustness screening model. The selection of desirability values was based on RSD from TPA, PR, and PA responses at optimum point, being defined 0% as desirable, 0.5% as acceptable, and 1.0% as unacceptable. The DA plots for interactions between CMPs, namely the ACT content vs column flow, ACT content vs column temperature, and column flow vs column temperature, are presented in Fig. 4 a, b, and c, respectively.

The highest desirability index was verified with an ACT content between 85 and 86%, a column flow between 290 and 300 µL min^{-1}, and a column temperature between 80 and 81 °C, being that all RSD values were below of 0.1%. The data verification of robustness screening results was completed by the agreement analysis between the predicted and observed values at optimal point. The results from data verification are summarized in Online Resource 10. The difference between observed values and predicted values is very low, being that 100% of observed values for all CMAs were within the 95% confidence interval, demonstrating a high robustness level at MODR optimal point.

The method control was based on establishment of system suitability limits by generating large amount of data (100 cases) through the Monte Carlo bootstrapping simulation at CMP optimal point into MODR, followed by application of capability analysis for estimation of residual errors from CMAs responses. The capability analysis for TPA, PR and PA are presented in Fig. 4 d, e, and f, respectively.

Most of cases (99%) from Monte Carlo bootstrapping simulation for TPA remained between the lower specification

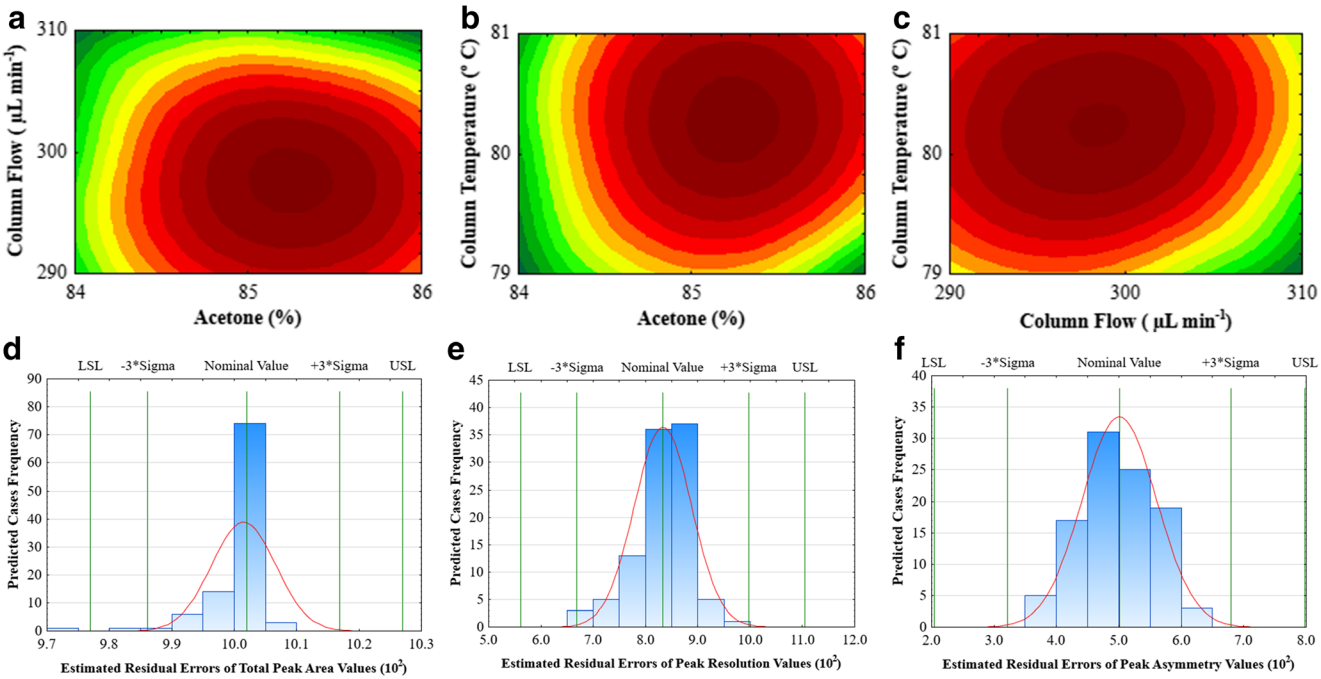

Fig. 4 Desirability analysis index plot of interactions between the continuous CMPs, ACT content vs column flow (**a**), ACT content vs column temperature (**b**), and column flow vs column temperature (**c**), for evaluation of robustness at optimum point into MODR according. Capability analysis plot regarding the total peak area (**d**), peak resolution (**e**), and peak asymmetry (**f**) residual error results

limit (0.098) and upper specification limit (0.103) established for residual error values (RSD < 0.1%). The process capability index (Cpk) for TPA was 2.01. For PR and PA response simulation, all cases (100%) were within the lower specification limit (0.056 and 0.020) and upper specification limit (0.083 and 0.080), where Cpk values were 1.70 and 1.60, respectively. The reference value of Cpk is 1.33, being the minimum value for a method to be considered robust (Tol et al. 2016). Thus, based on the obtained results from robustness screening and method control analysis at optimum point into MODR, our method demonstrated to have an exceptional robustness and prediction capacity.

Validation of the Analytical Method

The analytical method was validated for the following parameters: selectivity, calibration function, linearity, accuracy, precision, matrix effect, LOD, and LOQ, being performed according to guidelines from IUPAC (Thompson et al. 2002), and based on validation procedures established in our previous studies (Silva et al. 2017a, 2017b).

Selectivity evaluation was based on the analysis of chromatogram quality, namely the peak shape, resolution, RT, and the absence of interferences. The RTs are presented in Table 2. The chromatograms, (A) SCH sample spiked with sugars standards and (B) SCH sample, obtained at optimum conditions are presented in Fig. 5. The chromatograms showed that all sugars were clearly separated into a 20-min run time, and the peaks shape were a well-defined Gaussian. Moreover, our analytical method also demonstrated to have a high selectivity when applied to real SCH samples, where PR values of identified sugars were 3.0 (GLU), 4.2 (FRU), and 3.3 (SUC).

Calibration functions of GLU, FRU, SUC, XYL, and MAN were performed by construction of the standard calibration curves prepared with six concentrations levels, being plotted as TPA vs concentration level. For each calibration curve, the function model and correlation coefficient were determined. The linearity of calibration functions was verified by the suitability analysis of each function model through determination of Fisher variance (F test), where if the ratio $F_{\text{theoretical}}/F_{\text{experimental}}$ value is higher than 1.0, the function model is suitable to describe the observed data without significant lack of fit. The results are summarized in Table 2.

Two different function models, linear and polynomial, were proposed and tested to validate the suitability of calibration curve for each sugar under analysis. The correlation coefficients of calibration curves for all sugars presented values above 0.99 in linear and polynomial function models. However, the F test results demonstrated that the linear function model was not suitable ($F_{\text{theo}}/F_{\text{exp}} < 1$) for all sugars. Instead, the F test results confirmed that the polynomial function model was suitable to describe the TPA response vs concentration level for all sugars, where the values of ratio $F_{\text{theo}}/F_{\text{exp}}$ were always higher than 1. The evaluation of accuracy, precision, matrix effect, LOD, and LOQ was based on the described polynomial function models. The results are summarized in Table 3.

The accuracy was achieved by determination of recovery rate (%), being obtained by the ratio between the theoretical concentration value added to sample with experimental concentration values in a SCH sample spiked at low, medium, and high concentrations levels of each sugar. The analytical method proved to be strongly accurate at high-level concentrations. The recovery rates ranged between 98.2 (FRU) and 103.3% (MAN), wherein all recovery values at high-level concentrations remained within the recommended limits from literature guidelines (95–105%) (Gustavo González and Ángeles Herrador 2007). However, the accuracy was slightly inferior at medium and low-level concentrations, where some recovery values were above the recommended limits. These values may be due to the fact that the IR detector presents some noise at the chromatograms baseline. The precision was determined by repeatability and intermediate precision values, and were obtained through the inter- and intraday performance tests, respectively. The intraday variation was analyzed by evaluating ten replicates on the same day and inter-day variation by four replicates for each day during 3 days. The precision results were expressed as % RSD. The precision of analytical method was very satisfactory, the repeatability values ranged between 3.8 (GLU) and 6.4% (XYL) and the intermediate precision values varied from 2.1 (SUC) to 6.1% (FRU), where all obtained values were below the maximum reference value (RSD < 10%) (Gustavo González and Ángeles Herrador 2007). The matrix effect was determined by standard additions method, where the slopes from calibration curves of each sugar in H_2O:ACT solution and SCH sample were compared at same concentration levels. The appearance of significant differences between the slopes was statically evaluated by Student t test. The matrix effect results ranged between 91.76 (SUC) and 101.24% (MAN), wherein no significant differences were verified between slopes values from calibration curves, confirming that the matrix effect of analytical method was not significant. The LOD and LOQ were determinate based on standard deviations of interception and slope values from calibration curves performed in triplicate for each sugar, being calculated by following equations: $\text{LOD} = 3.3 \times s\ \text{slope}^{-1}$ and $\text{LOD} = 10 \times s\ \text{slope}^{-1}$. The LOD and LOQ values obtained through our analytical method were: 2.90 and 8.78 g kg^{-1} for GLU, 2.51 and 7.60 g kg^{-1} for FRU, 1.02 and 3.10 g kg^{-1} for SUC, 2.67 and 8.10 g kg^{-1} for XYL, and 3.24 and 9.81 g kg^{-1} for MAN, respectively. Comparing the LOD and LOQ values obtained for our analytical method with those described in the study performed by Xu and colleagues (Xu et al. 2015), where a method also based on LC-RI was developed for sugar analysis in sugarcane molasses samples, it was possible to confirm that the values obtained by our method are evidently lower to those

Table 2 Calibration function and linearity results

Sugars	RT (min)[a]	Concentration range (g l^{-1})	Linear					Polynomial				
			Regression equation	Correlation coefficient (r^2)	F_{exp} value[b]	Diference[c] F_{theo}/F_{exp}	Function suitability[d]	Regression equation	Correlation coefficient (r^2)	F_{exp} value[e]	Diference[f] F_{theo}/F_{exp}	Function suitability[d]
GLU	8.48	157.30–3.09	$y = 0.0968x + 0.4109$	0.9977	1.38	1.99	Yes	$y = -0.00004 \times 2 + 0.1031x + 0.2725$	0.9980	1.50	1.90	Yes
FRU	10.37	156.44–3.04	$y = 0.0859x + 0.3358$	0.9990	1.13	2.42	Yes	$y = 0.00002 \times 2 + 0.0895x + 0.2587$	0.9992	1.43	2.00	Yes
SUC	14.32	178.06–7.65	$y = 0.1256x + 0.2178$	0.9990	2.20	1.25	Yes	$y = -0.00004 \times 2 + 0.133x + 0.0023$	0.9993	1.67	1.71	Yes
XYL	7.51	160.13–4.36	$y = 0.0678x + 0.2748$	0.9976	6.99	0.39	No	$y = -0.00007 \times 2 + 0.0795x + 0.0117$	0.9996	0.38	7.61	Yes
MAN	9.40	158.66–4.26	$y = 0.0761x + 0.1035$	0.9996	0.74	3.69	Yes	$y = -0.00002 \times 2 + 0.0796x + 0.0281$	0.9998	2.26	1.26	Yes

[a] Retention time

[b] The Fisher value calculated experimentally with 16 degrees of freedom of experimental error and 6 degrees of freedom of lack of fit of function

[c] The Fisher value calculated experimentally is compared against the critical value of Fisher theoretical at the 95% with 16 degrees and 6 of freedom degrees. If the experimental data set describes a proposed function calibration of the form given then the condition $F_{theo.} > F_{exp}$ must be fulfilled

[d] The suitability of proposed function model to describe the observed data due to some significant lack of fit

[e] The Fisher value calculated experimentally with 16 degrees of freedom of experimental error and 5 degrees of freedom of lack of fit of function

[f] The Fisher value calculated experimentally is compared against the critical value of Fisher theoretical at the 95% with 16 degrees and 5 of freedom degrees. If the experimental data set describes a proposed function calibration of the form given then the condition $F_{theo.} > F_{exp}$ must be fulfilled

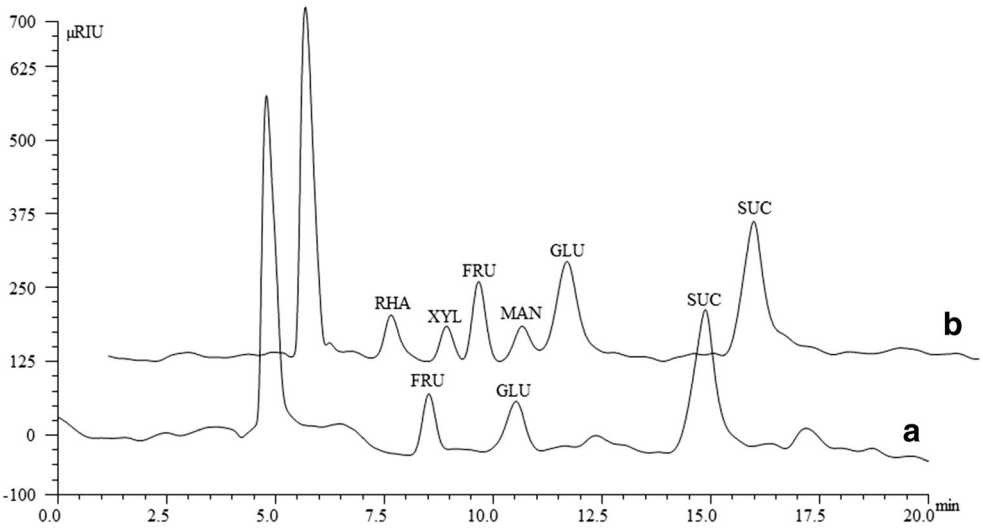

Fig. 5 Typical LC-RI chromatograms obtained for a sugarcane honey sample (**a**) and sugarcane honey sample spiked with sugar standards (**b**). Abbreviations bars correspond to (GLU) glucose, (FRU) fructose, (SUC) sucrose, (XYL) xylose, (MAN) mannose, and (RHA) rhamnose

described in the study by Xu and colleagues (Xu et al. 2015), which presented LOD and LOQ values of 5.41 and 27.09 g kg^{-1} for GLU, 9.91 and 49.49 g kg^{-1} for FRU, and 17.84 and 89.19 g kg^{-1} for SUC, respectively.

Method Application

The applicability of USA-LLE/LC-RI analytical method was verified through the analysis of GLU, FRU, SUC, XYL, and MAN in SCH samples from a certified producer obtained during three consecutive production years (2016, 2017, and 2018). The results for the method applicability are summarized in Online Resource 11. The mean concentration values plot is presented in Fig. 6.

Based on the obtained results, it was possible to confirm the applicability of the developed analytical method on SCH real samples. Moreover, the results also demonstrated its high repeatability when applied in real SCH samples, where the

Table 3 Figures of merit of the method: accuracy, precision, matrix effect, LOD, and LOQ

Sugars	Concentration range (g l^{-1})	Accuracy		Precision		Matrix effect		LOD (g kg^{-1}_{SCH})	LOQ (g kg^{-1}_{SCH})
		Recovery (%)	RSD (%)	Repeatability (% RSD)	Intermediate precision (% RSD)	Variation effect (%)	Significance (p value < 0.05)		
GLU	27.24[a]	101.4	2.8	3.8	3.4	92.94	0.54	2.90	8.78
	11.28[b]	110.8	4.9	6.2	6.1				
	4.63[c]	114.4	5.5	5.1	4.6				
FRU	26.81	98.2	3.0	5.6	2.5	95.52	0.55	2.51	7.60
	11.10	108.4	4.5	6.1	6.1				
	4.56	114.7	1.5	5.9	4.8				
SUC	67.36	99.9	4.1	4.1	2.1	91.76	0.36	1.02	3.10
	27.89	112.6	2.7	5.4	3.7				
	11.46	118.6	4.0	6.0	5.3				
XYL	27.23	98.3	2.6	5.1	3.8	93.97	0.09	2.67	8.10
	11.01	115.5	1.2	6.4	3.7				
	4.35	119.5	6.0	5.9	5.2				
MAN	26.62	103.3	1.2	4.8	2.8	101.24	0.84	3.24	9.81
	10.76	108.2	3.4	4.8	2.7				
	4.25	108.9	3.7	4.8	2.8				

The p value calculated experimentally through ANOVA test between slopes obtained by calibration curves of sugars in SCH sample and solvent. If the obtained p value was above that 0.05, no significant matrix effect was observed

[c] Low level concentration

[b] Medium level concentration

[a] High level concentration

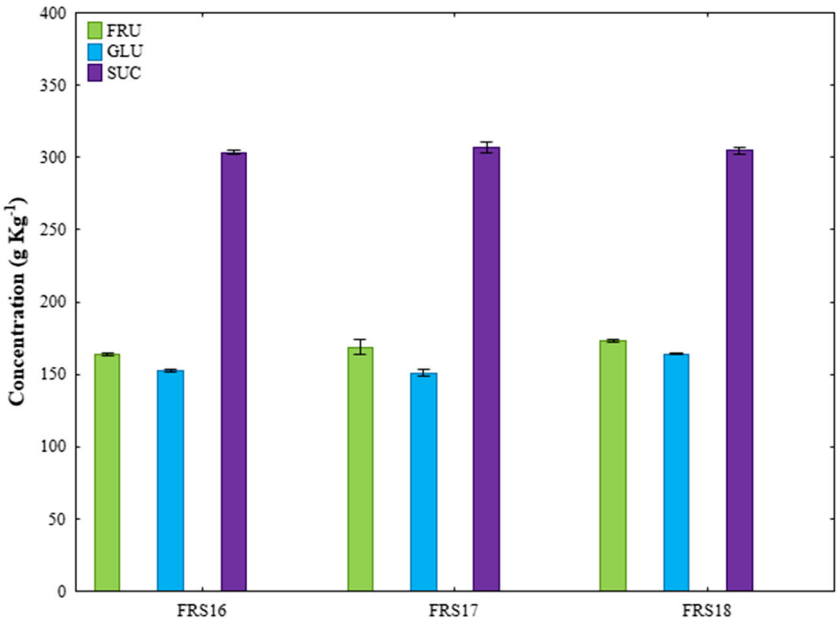

Fig. 6 The mean concentration values ($n = 3$) of sugars obtained by USA-LLE/LC-RI analytical method for the sugarcane honey samples. Abbreviations bars correspond to (GLU) glucose, (FRU) fructose, (SUC) sucrose, (FRS16) certified sugarcane honey sample from 2016, (FRS16) certified sugarcane honey sample from 2017, and (FRS18) certified sugarcane honey sample from 2018

highest RSD value was 3.2%, being that all concentrations values were obviously above the LODs and LOQs from the validation procedure.

Regarding the results from three SCH samples under analysis, only FRU, GLU, and SUC were identified and quantified in SCH samples under analysis, where the concentration values for FRU were 163.9 g kg^{-1} (2016), 168.9 g kg^{-1} (2017), and 173.4 g kg^{-1} (2018); for GLU were 152.8 g kg^{-1} (2016), 151.1 g kg^{-1} (2017), and 164.0 g kg^{-1} (2018); and for SUC were 303.5 g kg^{-1} (2016), 307.0 g kg^{-1} (2017), and 304.7 g kg^{-1} (2018). Apparently, and according to concentrations values of FRU, GLU, and SUC from samples of the three production years, the typical sugars ratio of SCH product was 1:1:2 (GLU:FRU:SUC).

Conclusions

A precise and robust USA-LLE/LC-RI analytical method was successfully developed according to AQbD approach for GLU, FRU, SUC, XYL, and MAN analysis in real SCH samples. According to results obtained from method scouting, the more efficient procedure for sugars extraction from SCH samples was based on LLE assisted by ultrasounds during 10 min, with an extraction solution composed by H_2O:ACT (50:50%, $v\ v^{-1}$) in a ratio sample/solution of 1:3 ($w\ v^{-1}$). The MODR was based on chromatography performance, wherein its optimal conditions for LC-RI analysis was obtained with a BEH AMIDE column operating at a temperature of 80 °C, flow rate of 300 μL min^{-1} and an eluent composed by H_2O:ACT (15:85%, $v\ v^{-1}$) in isocratic gradient mode. The method control procedure demonstrated through Monte Carlo simulation and capability analysis that the developed analytical method was highly robust at optimal point of MODR. The analytical method also proved through the validation procedures to be selective, accurate, precise, and without significant matrix effect, demonstrating lower LOD and LOQ values compared to other similar previous studies. The applicability of the method was confirmed with high repeatability by sugars analysis of real SCH samples provided by a certified producer during three consecutive production years (2016, 2017, and 2018), where only GLU, FRU and SUC were identified and quantified in typical ratio of 1:1:2, respectively. In conclusion, it is possible to state that the application of USA-LLE/LC-RI analytical method provide a simple, effective, precise and robust strategy for the establishment of typicity of genuine SCH product, being a useful and promising tool to guarantee its authenticity in a global market.

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Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Pedro Silva, Catarina L. Silva and Rosa Perestrelo. The first draft of the manuscript was written by Pedro Silva, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent is not applicable to this study.

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