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Identification of the rice syrup adulterated honey by introducing a candidate marker compound for Brown rice syrups

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ABSTRACT

Identification of honey adulteration is an important area to ensure product safety and quality. White rice syrups (WRS) or brown rice syrups (BRS) can be used for honey adulteration. Up to date, qualitative analysis of 2-acetylfuran-3-glucopyranoside (AFGP) and the quantification of the arsenic residue are the commonly preferred methods to detect rice syrups (RS). We have figured out the BRS may have very low amount of AFGP. Therefore, it was estimated that AFGP alone may not be a very reliable marker for BRS identification. We aimed at identifying a new marker compound for BRS and to develop a novel analytical method that allows simultaneous monitoring of this compound and AFGP to highlight the addition of RS from different origins. The characteristic molecule in BRS was identified as sorbic acid. A UHPLC-MS/MS method was developed by combining dilute & shoot sample pretreatment and 107 samples were analyzed. While 21 of the samples were found adulterated with BRS, 3 samples were found to contain WRS. We suggest using sorbic acid as a marker of BRS addition to honey. Within this research, it was hypothesized that fraudulent was mostly made with BRS and adulteration may be overlooked applying the existing methodology.

1. Introduction

The problem of fraud at food has increased considerably due to the economic concerns of producers and their efforts to make higher profits and the increment in the procurement prices of raw materials required for production. Honey is a kind of valuable foodstuff and in recent years, honey increasingly has been a target for adulteration considering its expensive price, production, and consumption levels. Depending upon the increased demand at consuming of bee products, leaning to fraud at honey commerce is a current worrisome. Honey is a very complex matrix and it includes plenty of nutrients such as carbohydrates, proteins, enzymes, organic acids, phenolics, minerals, and amino acids. The majority of these nutrients are monosaccharides such as glucose and fructose, other highly abundant small and large molecules may be in variable amounts due to honey botanical origin differences and seasonal variations (Machado De-Melo, Almeida-Muradian, Sancho, & Pascual-Maté, 2018; Santos-Buelga & González-Paramás, 2017). This situation makes the natural honey matrix very tough to select a suitable and reliable adulteration marker compound from it. The complexity of the honey matrix allows it to be manipulated by different sugar syrups at low percentages since these amounts are unnoticeable to be detected. Possible forms of adulteration such as mixing honey with inexpensive sugars or sugar syrups may also adversely affect the composition and therefore the quality of honey (Chen et al., 2019; Soares, Amaral, Oliveira, & Mafra, 2017). According to current regulations, honey is considered as the natural sweet substance obtained from the nectar or secretions of the living parts of plants and is never modified by additives, colorants, conservatives, or other substances (Commission, 2001, pp. 19–26). Honey adulteration can be directly by adding a substance to honey or indirectly by feeding of honeybees with fraudulent substances (Cordella et al., 2002; Rios-Corripio, Rojas-López*, & Delgado-Macuil, 2012). Typically, honey is adulterated with cheaper sweeteners including high fructose corn syrup (HFCS), sucrose syrup, maltose syrup, brown rice syrup (BRS), white rice syrup (WRS), beet syrup, golden syrup, treacle syrup, glucose syrup, maple syrup, as well as industrial grade sugars like glucose and fructose. Among the mentioned

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adulteration substances, syrups produced from starch can often be used for the adulteration of honey, and these could be obtained from starch following the enzymatic or acid treatment (Amiry, Esmaiili, & Alizadeh, 2017; Geana & Ciucure, 2020; Wu et al., 2017). This type of adulteration is difficult to detect owing to the fact that similarities in the sugar composition between these low-cost syrups and natural honey (Cordella, Militao, Clément, Drajnudel, & Cabrol-Bass, 2005; Cotte, Casabianca, Chardon, Lheritier, & Grenier-Loustalot, 2003). On the other hand, the rising demand for honey in the market has made it definitely necessary to establish reliable methods of analysis in order to provide authenticity. Therefore, effective and feasible analytical methods to detect adulterated honey are indispensable. There is a number of studies in the literature that present plausible adulteration markers or analysis methods such as quantification of AFGP molecule along with arsenic as rice syrup markers (Jackson, Taylor, Karagas, Punshon, & Cottingham, 2012; Xue et al., 2013), oligosaccharide screening (Arias, Castells, Malacalza, Lupano, & Castells, 2003), detection of difructose anhydride molecule (Montilla, Ruiz-Matute, Sanz, Martínez-Castro, & Del Castillo, 2006), mercury residue (Toth, Kopernická, Sabo, & Kopernicka, 2016), and C4-% (SCIRA) analysis for cane and corn syrup identification (Cabanero, Recio, & Ruperez, 2006; Simsek, Bilsel, & Goren, 2012). There are also enzymatic-based assays named as foreign enzyme determinations to detect adulteration and to name but a few are foreign diastase or heat-stable diastase, foreign invertase and, beta/gamma amylase (Voldřich, Rajchl, Čížková, & ; Wang et al., 2015). Most of the methods in the literature can detect the presence of a single type of sugar syrup in honey thus a couple of analytical approaches should be applied sequentially for the complete identification of honey adulteration. Several instrumental analytical techniques are used for honey quality control and the detection of suggested honey adulterants as the targeted approach is accomplished by applying different chromatography incorporated techniques such as gas chromatography (GC) (Ruiz-Matute, Rodríguez-Sánchez, Sanz, & Martínez-Castro, 2010; Ruiz-Matute, Soria, Martínez-Castro, & Sanz, 2007), thin-layer chromatography (TLC) (Puscas, Hosu, & Cimpoiu, 2013), high-performance liquid chromatography (Wang et al., 2015), elemental analysis carbon isotope ratio mass spectrometry (Cabanero et al., 2006; Padovan, De Jong, Rodrigues, & Marchini, 2003). Additionally, there are spectroscopic non-targeted-based approaches including infrared spectroscopy (IR) (Ferreiro-González et al., 2018; Latorre, Crecente, Martín, & García, 2013), nuclear magnetic resonance (NMR) (Bertelli et al., 2010), and fluorescence (Dramićanin, Lenhardt Acković, Zekovi). The vast majority of these methods are routinely applied in the honey commerce by responsible laboratories but these analytical methods have also some limitations. At this point, we focused on the revision of the aforementioned methods that used to detect the addition of rice syrups in honey samples. Rice syrup, originating from the C3 plant, is an emerging adulterant from the hydrolysis of oligosaccharides and polysaccharides in rice and thus follows a similar Calvin cycle of photosynthesis as the plants from which natural honey results, making it difficult to detect (Xue et al., 2013). In literature, there is a limited number of researches based on the determination of the honey adultered with rice syrups. Recently, qualitative analysis of 2-acetylfuran-3-glucopyranoside (glucosylisomaltol, AFGP) molecule to detect rice syrup adulterations is the commonly preferred method for both research and routine analysis purposes (Xue et al., 2013). Besides, arsenic (As) quantification by means of the Inductively coupled plasma-mass spectrometry (ICP-MS) may also be seen for both identification and confirmation (Jackson et al., 2012). We have figured out the BRS has very low and varying amounts of AFGP in comparison to WRS. In this respect, it is possible to misinterpret and reporting false negative results if the adulteration was made with BRS. Within this actual work, we aimed at identifying a new marker compound for BRS using ultra-high-performance liquid chromatography with photodiode array detection (UHPLC-PDA) and to develop a novel, intuitive, and versatile analytical method based on ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) that allows simultaneous monitoring of this compound and AFGP to highlight the addition of rice syrups from different origins. It was also purposed to investigate the prevalence of rice syrup adultered honeys in the market by applying this novel analytical method and recent rice syrup markers (arsenic and AFGP) were tested in terms of rice syrup identification accuracy. To the best of our knowledge, this research contributes to the literature as the first method that can monitor the BRS adulteration in honey concomitantly with WRS adulteration.

2. Material and methods

2.1. Sample collection

The authentic honey samples of different origins were gathered from apiaries directly by researchers in Turkey and classified according to their melissopalynology and authenticity assay outcomes. A total number of 20 honey samples (blossom (10), pine (6), and chestnut honey (4)) were chosen to investigate as unadulterated blank matrices and their melissopalynology analysis was accomplished to confirm their botanical origins. To ensure the authenticity of the honey samples, several analyses such as foreign invertase (β-fructofuranosidase activity), foreign amylase, heat-stable amylase, β/γ amylase, C-4 sugar test (SCIRA), starch content, and physicochemical quality assays like conductivity, pH, sugar profile, proline content, and humidity test were performed. These samples were used both to mimic adultered honey by addition of the rice syrups and for investigating the natural presence of the rice syrup characteristic compounds in genuine honey samples. A total number of 107 different commercially available honey samples were purchased randomly from some markets in Turkey including more than 15 different provinces in the season of 2021. These samples were analyzed to perform real sample application of the developed method and to see the frequency of fraud with rice syrup in honey trade. Collected 107 samples were classified according to their botanical origin. According to this, 14 pine honey, 4 chestnut honey and 89 blossom honeys were analyzed. Samples were taken in glass jars, sealed hermetically, and stored at 4 °C until analysis.

2.2. Preparation of adultered samples

Representative BRS (2) and WRS were purchased and used for the characteristic molecule investigation but also for the preparation of the non-authentic honey samples. In order to verify the adulteration identification capability of the novel developed method, the simulation of manipulated honey was made by mixing certain amounts of WRS and BRS with genuine honey samples. In this context, honey samples containing 10%, 20%, and 40% (w/w) BRS and WRS were prepared separately.

2.3. Reagents and chemicals

HPLC grade formic acid, acetic acid, ammonium formate, ammonium acetate, ethyl acetate was purchased from Sigma-Aldrich® (St. Louis, MO, U.S.A.). ACS grade acetonitrile (ACN) and methanol (MeOH) were supplied from VWR (Radnor, PA, U.S.A.). Sodium sulfate anhydrous (Na_2SO_4), and sodium chloride were from EMD Chemicals (Gibbstown, NJ, U.S.A.). Analytical standards of sorbic acid, 2-acetylfuran-3-glucopyranoside (AFGP) and methyl *p*-hydroxybenzoate as internal standard (IS) were supplied from Dr. Ehrenstorfer® GmbH (Bürgermeister-Schlosser-Straße, Augsburg, Germany). The purity of all the analytical standards was above 98.8%. Deionized water was obtained from Milli-Q Plus® from Millipore® (Bedford, MA, U.S.A.) and was used at all dilutions.

2.4. Sample pretreatments

2.4.1. Modified Swedish ethyl acetate extraction (MSweEt) procedure

Previously, Ekroth (2011) described the SweEt method which is based on ethyl acetate extraction of pesticides was modified and named as MsweEt within this study to both extract and enrich the unknown molecule. For this, 30 g of BRS was weighed into 6 separate 50 mL falcon tubes. A relatively high amount of BRS was weighed (180 g) and to improve the extraction efficacy. 15 mL of distilled water was added to each. NaCl and Na_2SO_4 salts were consecutively added to the solutions at a final concentration of 2g/100 mL for each. Samples were thoroughly homogenized by vortexing. Then, 12 mL of ethyl acetate was added to these admixtures as extraction solvent and the samples were agitated for 30 min in a horizontal shaker. After the extraction step, the samples were spun in a centrifuge for 10 min at 4000×*g*. The supernatants were combined into a separate falcon tube. For further concentration and solvent exchange steps, ethyl acetate supernatant was evaporated to dryness under a stream of nitrogen and the dry pellet was reconstituted by using 1.5 mL of 35% MeOH solution. Next, the final solution was filtered into a vial and a large volume of sample (250 μL) was injected into the MPLC system manually. By using this approach, the candidate molecule could be isolated at the appropriate concentration for identification. Alternatively, the MSweEt sample preparation was repeated in the same manner, and the derivatization step was utilized after nitrogen evaporation. The molecule was tried to be identified by injecting TMS-derived and non-derivative extract into the GC-MS system. After MSweEt preparation, 250 μL of pyridine (Sigma-Aldrich®, Munich, Germany), and 300 μL of BSTFA:TMCS (N,O-Bis(trimethylsilyl)trifluoroacetamide, with 10% TMCS - Alfa Aesar™, Erlenbachweg, Germany) were added to the dried sample and the residue was dissolved by vortexing for 1 min. After the sample was transferred to a glass vial, it was left to incubate at 80 °C for 10 min. After incubation, this sample was also injected into the GC-MS system.

2.4.2. Dilute & shoot protocol

For sample pretreatment of both final UHPLC-MS/MS and untargeted UHPLC-PDA screening methods an optimized dilute & shoot protocol was developed (Seraglio et al., 2016; Valese et al., 2016). For this, 2 g of honey or syrup sample was weighted and 10 mL of deionized water was added in a 15 mL centrifuge tube. 100 μL of methyl *p*-hydroxybenzoate in 100 mg/L (in MeOH) concentration was spiked as an analog internal standard prior to homogenization. The sample was vortexed until homogenization. The resulting supernatant was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) syringe filter (Interlab®, Arnavutköy, Istanbul, Turkey). The permeate was taken in an amber vial and 50 μL for targeted UHPLC-UV and 20 μL for UHPLC-MS/MS was injected. A stock standard mixture standard of AFGP and sorbic acid (10 mg ± 0.05 mg) were prepared by dissolving them in 10 mL of MeOH. A working standard mixture of 10 mg/L was prepared using water as a diluent and adequate amounts of this solution were spiked to the blank honey to complete matrix-matched linearity, intra-day and inter-day precision, and limit of detection (LOD) & limit of quantitation (LOQ) studies.

2.5. Instrumental conditions

2.5.1. Untargeted UHPLC-PDA and targeted UHPLC-UV analysis

Untargeted UHPLC-PDA analysis was conducted using Thermo Scientific® Accela UHPLC system (Waltham, Massachusetts, USA) equipped with an Accela 600 pump, an Accela autosampler, a column oven, an Accela PDA detector, and an ACE® C18-PFP (250 × 4.0 mm, 5 μm) column. Instrument control and data acquisition were carried out using Chromquest® 3.1 software. Compounds were chromatographically resolved using gradient elution and mobile phases consisted of mobile phase (MP)-A (water, 0.5% (v/v) formic acid) and consisting of MP-B (MeOH, LC grade) at a flow rate of 0.45 mL/min. The run time of the screening method was 120 min. The linear gradient consisted of 90% MP-A and 10% MP-B for the first 1 min. MP-B increased to 45% methanol over 65 min and it was then increased to 90% over 100 min and held for 5 min. Finally, MP-B was reduced to 10% over 1 min and held for 14 min. The injection volume was 50 μL and the column temperature was maintained at 40 °C. The untargeted (screening) approach was performed first. Thereby wavelength range was set between 200 nm and 500 nm as screening mode at the PDA detector. Following the determination of the characteristic compound, 260 nm was chosen as a fixed wavelength at targeted UHPLC-UV and MPLC methods to get the best response since the molecule has a maximum absorbance at this value. The other UHPLC parameters were applied in the same manner as the scan mode without any changes when adapting targeted UHPLC-UV analysis. The targeted UHPLC-UV method was used to evaluate the purification efficiency of the MPLC analysis by injecting the fraction of interest into the UHPLC system.

2.5.2. Preparative liquid chromatography method (MPLC method)

Isolation of the concentrated marker component was achieved on a MPLC instrument named NGC Quest 10 middle pressure chromatography system from Bio-Rad® Laboratories, Inc. (Hercules, CA, USA) consisting of a preparative pump equipped with a multiple wavelength UV detector and conductivity detector. NGC fraction collector was also operated to obtain to the fraction of interest. A semi preparative column Kromasil® 100 C18, 5 μm, 10 × 250 mm (Nouryon®, Bohus, Sweden) was used during purification. The flow rate was set to 2.5 mL/min, the injection volume was 250 μL. The mobile phase compositions, gradient profiles, run time, and detection wavelength were the same as those used in targeted UHPLC-UV analysis. ChromLab® software from Bio-Rad® Laboratories, Inc. was used for the monitoring marker compound and the eluted desired molecule was analyzed by using the targeted UHPLC-UV method to verification of the purification efficacy. The purified compound was kept in the refrigerator at −20 °C until analysis and further investigation to identification was accomplished by injecting the fraction into the Mass spectrometry coupled to gas chromatography (GC-MS) system.

2.5.3. GC-MS compound identification analysis

The fraction collected from MPLC and whose purity was confirmed by analysis was injected both directly and after BSTFA:TMCS derivatization into the GC-MS system. For GC-MS identification analysis, the inlet was operated in splitless mode (280 °C, 2 μL injection) to get a higher response at Agilent® 7890B GC system equipped with Agilent® 7693A Automatic Liquid Sampler (ALS) with G4513A injector (Agilent®, Santa Clara, CA, USA). Compounds were chromatographically separated by using Agilent® J&W HP-5MS capillary column coated with a 5% phenyl-methyl siloxane/95% methyl polysiloxane stationary phase (30 m, 0.25 mm i.d., 0.25 μm phase thickness). Temperature gradient was utilized and helium was used as carrier gas at 1.2 mL/min. Electron Impact (EI) ionization at 70 eV as high energy ionization was employed for reaching the most ideal structural elucidation at Agilent® 5977A MSD detector. MS transfer line temperatures were maintained at 300 °C. The oven temperature was held at 80 °C for 2 min, raised to 250 °C at 100 °C/min and as a final operating temperature was raised to 320 °C at 3 °C/min and held for 1 min. MS acquisition was operated in full scan mode for identifying the target compound by comparing its mass spectrum with embedded The National Institute of Standards and Technology (NIST) 11 (version 2.0) electron ionization (EI) mass spectrum library. NIST′11 Mass Spectral Library with >243,000 spectra was employed with the scan range from 35 to 550 amu at up to 12,500 μ/s. Match factor, reverse match factor, and probability (%) was calculated associated with the selected compound. These values were used to form a hit list of compounds that NIST has identified as possible matching compounds.

2.5.4. UHPLC-MS/MS parameters

The mass spectrometry technique was preferred at the final determination protocol by using a Waters® ACQUITY UHPLC (Waters®, Milford, MA, USA) system equipped with a binary solvent delivery system and autosampler. The chromatographic resolution was achieved by means of Waters® Acquity UHPLC Cortecs T3 column (2.1 mm × 150 mm, 1.6 μm). The mobile phase consists of solution A (water containing 0.005% formic acid) and solution B (ACN containing 0.005% formic acid). The gradient elution was optimized as follows: 5% B (0–1 min), 5–90% B (1–6.30 min), 90–5% B (6.30–6.31 min), 5% B (6.31–8 min). The flow rate was 0.3 mL/min. The column and autosampler temperatures were maintained at 40 °C and 4 °C, respectively. The injection volume was 20 μL and total run time was 8 min. The MS data was acquired via Waters® Xevo TQ tandem quadrupole mass spectrometer (Micromass® MS Technologies, Manchester, UK) with electrospray ionization (ESI) interface in multiple reaction monitoring (MRM) and employing both positive and negative ionization modes. The needle capillary voltage was set at 1.5 kV. The flow rate and temperature of desolvation gas were 850 L/h and 450 °C, respectively. Source temperature was operated at 150 °C and the flow rate of cone gas was 50 L/h. These conditions were optimized by analyzing the sorbic acid, methyl *p*-hydroxybenzoate and AFGP individually and by examining the signal-to-noise ratios (S/N) for each analyte. All ESI and mass spectrometer conditions were listed in Table 1. Data acquisitions and quantifications were performed using Waters® Mass-Lynx software with the Target Lynx program and all dwell times for ions were set automatically by the software (Table 1 near here).

2.5.5. ICP-MS arsenic analysis

The arsenic contents of the samples were determined by modifying the ICP-MS method (Kılıç, Dinç, Paksoy, . ICP-MS NexION® 350 (PerkinElmer, Waltham, MA, USA) equipped with a concentric nebulizer, glass cyclonic spray chamber, a quartz torch, Standard nickel-cones, and an octopole reaction system was used for the measurements. High-purity argon and helium (99.9995%) were used during analysis. The operating conditions were as follows: Plasma gas flow was 18.0 L/min, auxiliary gas flow was operated at 1.2 L/min, nebulizer gas flow was set to 0.98 L/min, sample uptake rate was 300 μL/min, RF power was used at 1600 W, number of replicates for per sample was 3 and, Universal Cell Technology™ KED mode was used. MARS 6®, microwave digestion system (CEM®, Matthews, NC, USA) with high-pressure vessels (CEM®, Matthews, NC, USA) made of TFM™-PTFE was used for sample digestion. To prevent contamination, all glassware and plasticware were 10% acid-washed before use. Nitric acid (Suprapur®, Merck, Darmstadt, Germany) was used for digestion. The calibration curve was assigned using standard prepared by diluting a stock solution of As. As standard stock solution at 100 μg/mL, (Inorganic Ventures®, USA) was used to prepare the external calibration curve (from 0.1 to 100 μg/L). A stock solution (800 μg/L) containing yttrium (Inorganic Ventures®, USA) was chosen as the internal standard and spiked into every sample prior to digestion. For sample preparation honey and rice syrup samples (0.5 g) were weighed into a digestion vessel. Reagent blanks were also prepared by the addition of water instead of the sample. A volume of 5 mL of concentrated HNO_3 solution was added to each digestion vessel. Samples were sealed, placed in the microwave, and digested. Afterward, they were transferred quantitatively into acid-washed polypropylene tubes, ten-fold dilution was applied to the final volume of 25 mL using 5% HNO_3, and stored at room temperature until analysis.

3. Results and discussion

3.1. Screening and selection of the candidate compound as adultertion marker

In this study, a favorable molecule to consider as an adulteration marker for BRS addition was demonstrated. Initially, as a compound selection pilot screen research, BRS was compared with natural honey samples by analyzing them at UHPLC-PDA instrument. As a result of the untargeted primary screen analysis, an unknown UV active compound was detected in BRS at about 54.8 min retention time. This molecule was also confirmed at another BRS sample. For the potency assessment of this candidate, genuine honey samples from different origins (chestnut, blossom, pine) were analyzed in parallel. It was also observed that all authentic honey samples from different origins do not include this unknown compound. Therefore, following identification and method development studies were planned to be able to identify solely this molecule, and the compound was selected as an appropriate BRS marker. The mentioned molecule had a UV absorption maximum at 260 nm hence this wavelength was chosen as an adjusted wavelength for targeted mode analysis and the method was switched as targeted UHPLC-UV analysis. This method was then used for the purity check of the isolation process. An illustration of the UHPLC - PDA screening comparison is given in Fig. 1 (Fig. 1 near here).

3.2. Enrichment and purification of the marker molecule

After determining the candidate molecules in the UHPLC-PDA system, it was necessary to obtain the most appropriate compound at a higher concentration in order to be able to identify it accurately. For identifying the unknown compound, a fractionation was made during untargeted UHPLC-PDA analysis. The collected fraction that includes the compound of interest was analyzed at the GC-MS system for identification. To being sure that the molecule is amenable to analysis by GC technique, both direct injection and BSTFA:TMCS derivatization was utilized to purposed effluent. Unfortunately, the obtained fraction from untargeted UHPLC-PDA analysis was in highly diluted form and it was anticipated that the marker compound was not at the convenient concentration for proper GC-MS detection due to unsatisfactory identification. Since the low amount seemed to be problematic, we intended to follow a couple of analytical procedures to enrich and isolate the molecule of interest by implementing MPLC isolation followed by MSweEt protocol. For this purpose, the option of purification via MPLC system, which allows injection of higher volumes was evaluated. Unfortunately, during purification, the molecule of interest would become somewhat diluted and the resulting fraction had to be concentrated as much as possible. In this regard, prior to the MPLC study, the sample preparation processes for BRS had to be capable of reducing the matrix effect and enriching the compound. At the elution time frame, the mobile phase was approximately at 35% MeOH composition and for this reason, it was evaluated as the compound has a mid-polar structure. Therefore, ethyl acetate was preferred as an extraction solvent which is a remarkably suitable solvent for the extraction of mid-polar molecules. NaCl and Na_2SO_4 admix was induced the salting-out effect, thereby an acceptable recovery was obtained during ethyl acetate extraction. With

Table 1
Selected Reaction Monitoring (SRM) transitions and mass spectrometric parameters selected for AFGP and sorbic acid determination in honey by UHPLC-MS/MS.

Compound	Retention time (min)	Precursor ion $[M+H]^+$ (m/z)	Product ions (m/z) (Q/q1/q2)	Collision energies (eV) (Q/q1/q2)	Cone voltages (V)
AFGP	2.76	311.3	149.5/185.4	15/15	30
Sorbic acid	4.46	113.2	41.0/67.0/95.0	20/20/10	25
Methyl *p*-hydroxybenzoate	4.59	151.0	92.0/136.0	25/20	20

Dwell times were 0.005 s, m/z ions used for quantification (Q) and for confirmation (q1, q2).

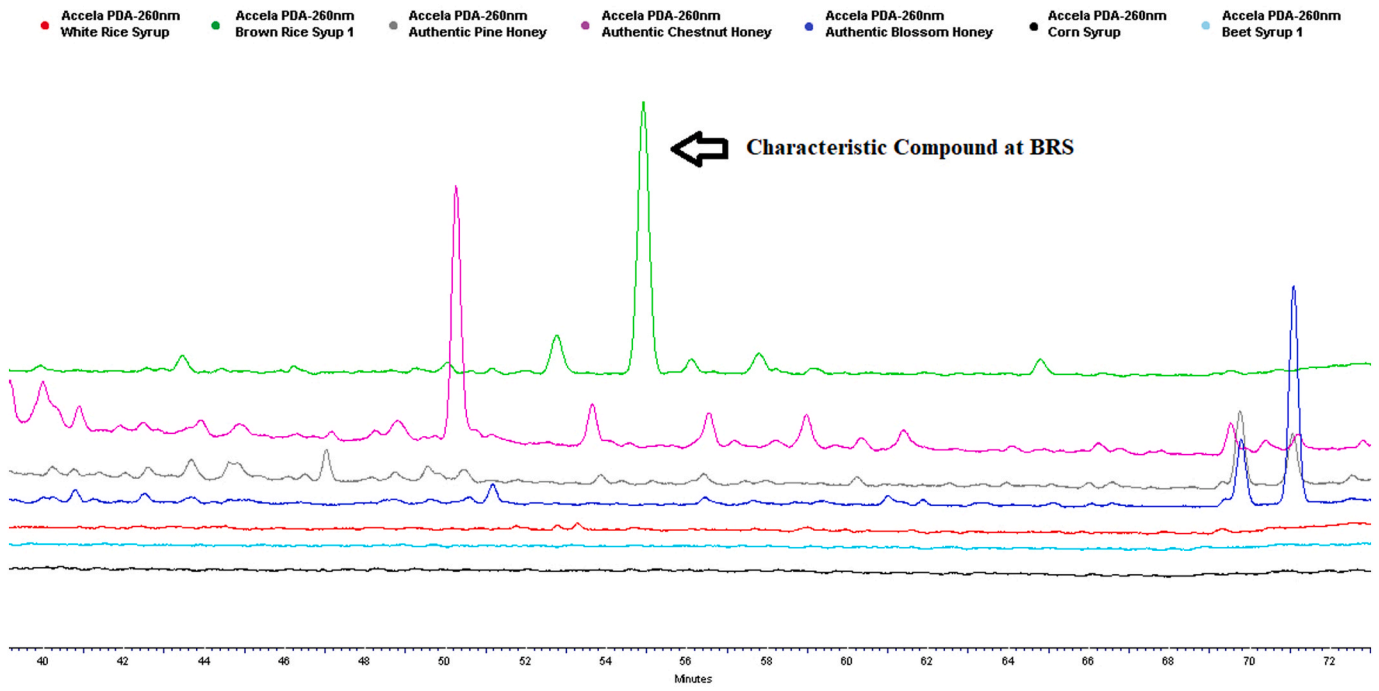

Fig. 1. Comparison of the UHPLC-PDA analysis profiles of the BRS, WRS, syrup samples and authentic honey samples from different origins.

the extract obtained after sample preparation, the marker compound was concentrated, cleaned up slightly from its matrix environment, and could be isolated easier at the MPLC system with balancing the dilution effect. The purity was checked by reinjecting the fraction of interest to both MPLC and targeted UHPLC-UV systems. The representative chromatograms before and after the purification approach are given in Fig. 2 as MPLC chromatograms (Fig. 2 near here).

3.3. Identification of the characteristic molecule

Identifying uncharacterized molecules in food samples is a significant analytical challenge owing to complex matrices and it requires perfectly defined analytical approaches. The mass spectrum matching is the widely used approach for compound identification in GC-MS. A well-resolved peak and high signal-to-noise ratio (S/N) in GC-MS is crucial for the unambiguous identification of a new marker entity. Therefore enrichment and following purification processes simplified the

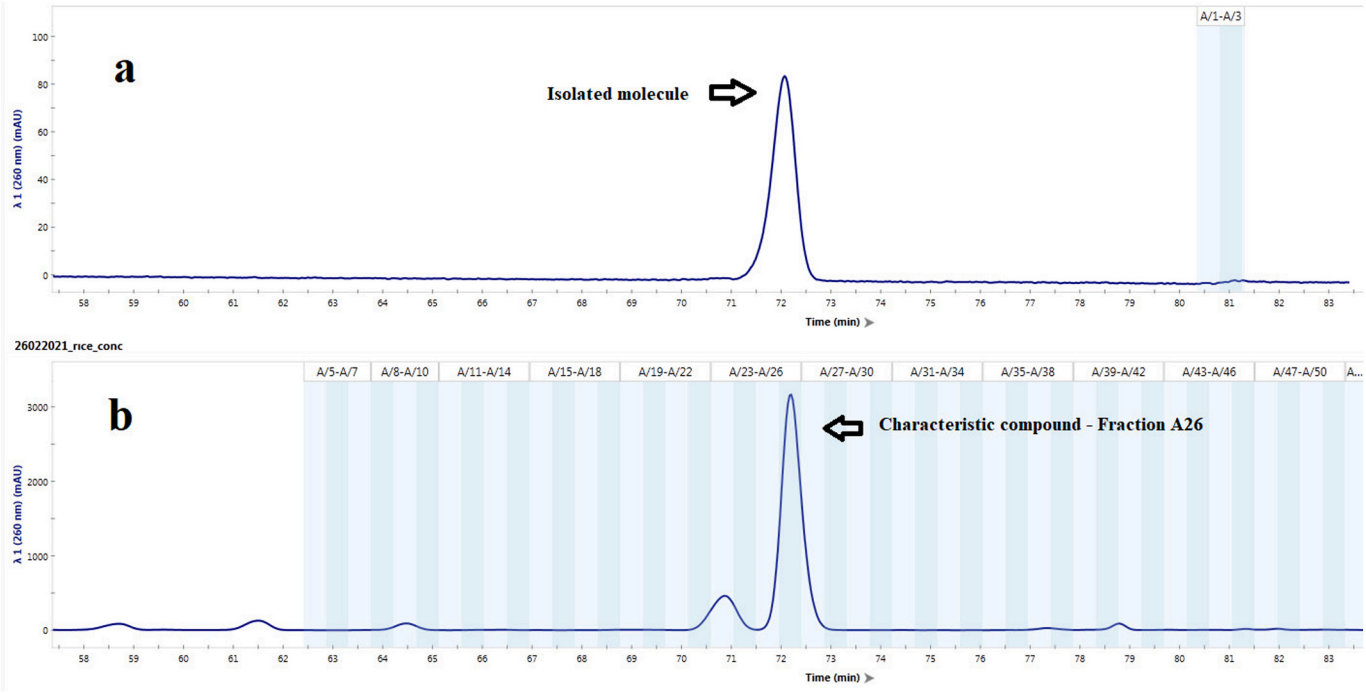

Fig. 2. Representative chromatograms of the purified molecule obtained at MPLC system. a) Chromatogram obtained by re-injection of the fraction obtained after purification into the MPLC system for control purpose. b) MPLC chromatogram of the solution obtained after MSweEt sample preparation.

identification process without the need for additional matrix clean-up or improved chromatographic resolution at the GC system. In this study, we performed compound identification using solely mass spectrum information of the isolated compound. The fraction collected from MPLC and whose purity was confirmed by analysis was injected both directly and after BSTFA:TMCS derivatization into the GC-MS system. The obtained mass spectral data was analyzed against the NIST reference mass spectral library. The compound of interest was eluted from GC capillary column at 3.67 min. TMS derivate of the fraction gave the appropriate signal and mass spectrum whereas the direct injection of the fraction was not successfully identified due to not enough signal was observed. According to the results, the unknown compound was identified as sorbic acid (2,4-hexadienoic acid, short-chain fatty acid) with a probability score of 80%. The obtained spectrum was compared and validated using a reference standard of sorbic acid as well. Identified mass spectrums of the sorbic acid molecule and its NIST reference spectrum were compared and the illustration of the mass spectral comparison is given in Fig. 3 (Fig. 3 near here).

3.4. UPLC-MS/MS method optimizations for simultaneous determination of AFGP and sorbic acid

An effective UHPLC-MS/MS method was developed for the simultaneous determination of AFGP and sorbic acid. Dilute & Shoot sample preparation was utilized prior to injection of the samples and the optimum chromatographic conditions on the basis of flow rate, column temperature, injection volume, mobile phase composition, LC gradient, the analytical column of choice was optimized to get the highest resolution and the best sensitivity. MS acquisition parameters were also optimized for each molecule by using the MS in scan mode and the most abundant quantitation and confirmation ions were selected. At least one confirmation ion was established for each compound to get confident outcomes. The optimum cone voltages and collision energies were found experimentally and MS/MS acquisition table was generated. Waters® Acquity UHPLC the Cortecs T3 column (2.1 mm × 150 mm, 1.6 μm) was chosen depending upon the different polarities of the target compounds. Superficially porous particles grafted with aqua-based C18 ligands exhibited reasonable retention times with narrower peak widths. 150 mm length was enough to achieve good resolution. Methyl *p*-hydroxybenzoate was used as an internal standard and a normalization approach was employed. Methyl *p*-hydroxybenzoate molecule was chosen as analog internal standard owing to the absence in the honey matrix but also its water solubility, ionization efficacy, and chromatographic retaining similarities to sorbic acid. 20 μL injection volume was preferred and this volume gave satisfying peak bandwidths with low LOQ rates without introducing the excess matrix effect. To keep the run time short, as elution mobile phase (phase B), ACN was used at 0.3 mL/min flow rate, since MeOH and lower flow rates delayed the elution time of sorbic acid. 0.3 mL/min flow rate also gave acceptable back-pressure for a relatively long 150 mm UHPLC column. The gradient was optimized to get superior resolution in a shorter elution timeframe and formic acid was used as a mobile phase additive. Acetic acid, formic acid, and additional volatile buffers such as ammonium format and ammonium acetate in variable concentrations were also tested but the best performance was obtained with the addition of formic acid as proton donor at 0.005% concentration. 1.5 kV needle capillary voltage, 850 L/h desolvation gas flow, and 450 °C desolvation temperature were the best conditions for ionization efficiency according to empirical optimization results. Dilute & shoot technique was used to facilitate the sample preparation steps of the method. Therefore, the embedded valve on the MS instrument was operated and hold in the waste position until 1.8 min at the beginning of the method to get rid of polar matrix interferences. This approach was able to keep the ion source clean and allowed the straightforward dilute and shoot procedure to be implemented. Fig. 4. Illustrates the obtained chromatograms from UHPLC-MS/MS analysis for the simultaneous determination of the AFGP and sorbic acid in the honey samples (Fig. 4 near here).

3.4.1. Method performance

To get reliable results, a validation study was also performed. Six matrix-matched standards of AFGP and sorbic acid were prepared by spiking the working standard mixture (10 mg/L) to a blank honey

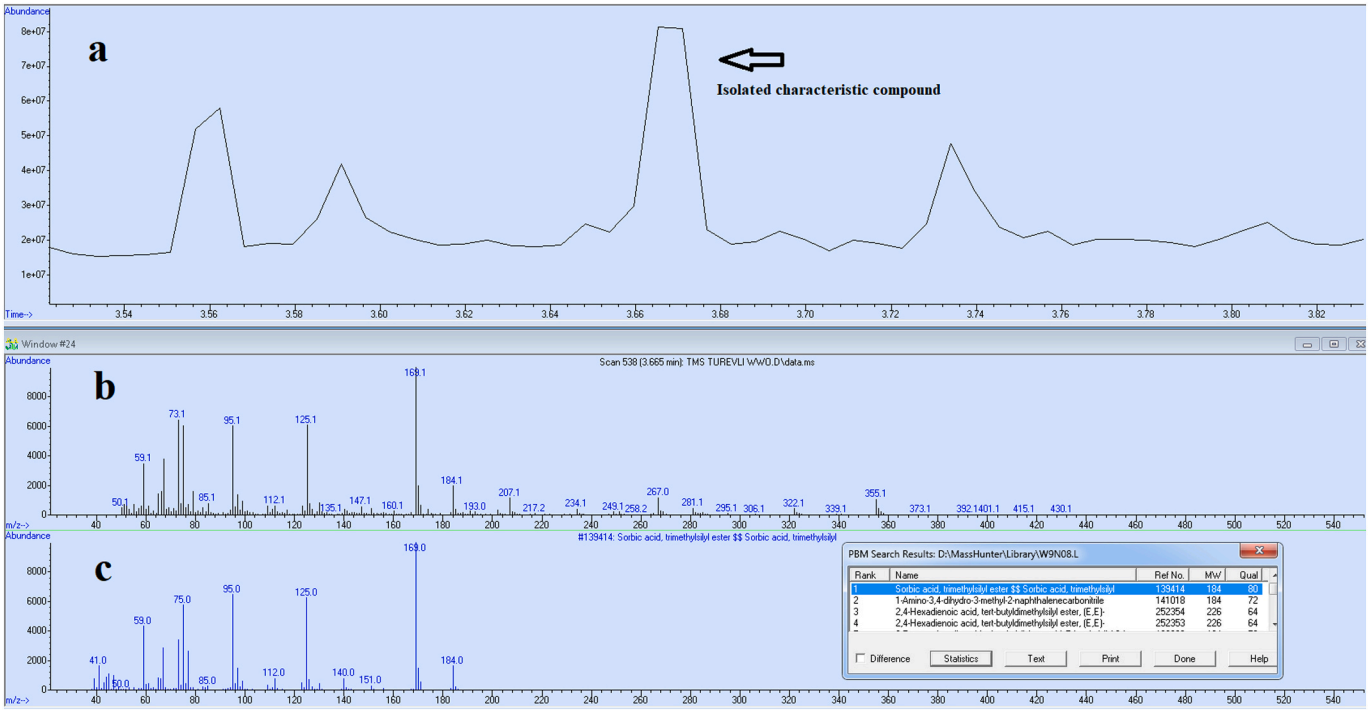

Fig. 3. GC-MS chromatogram and corresponding spectrum of the MPLC fraction. a) Chromatogram of the MPLC fraction, b) Mass spectrum of the MPLC fraction, c) NIST reference spectrum for sorbic acid.

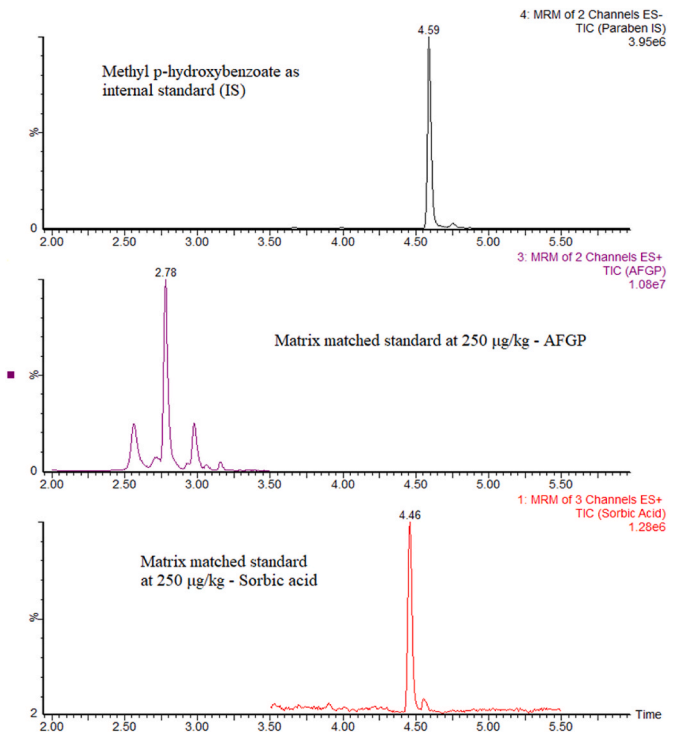

Fig. 4. TICs of the UHPLC-MS/MS analysis belongs to the sorbic acid and AFGP compounds in honey at the concentrations of 250 µg/kg.

sample that encompasses the (25, 50, 100, 250, 500, and 1000 µg/kg) concentrations. All matrix-matched standards were prepared according to dilute & shoot procedure and analyzed by UHPLC-MS/MS system. Both sorbic acid and AFGP standard curves had good linearity in the range of 25–1000 µg/kg (For sorbic acid; $R^2 = 0.9997$, and for AFGP; $R^2 = 0.9995$). For the precision study of AFGP and sorbic acid, intra-day precision (repeatability) and inter-day precision (intra-laboratory reproducibility) studies were conducted at three different concentrations (25, 100, and 1000 µg/kg) as seven replicates of each for both in the same day and for consecutive days. For AFGP molecule, RSD <2.7% and RSD <3.5% values on average were obtained for intra-day and inter-day precision studies consecutively. These values were calculated as RSD<2.8% and RSD <3.3% on average at sorbic acid for intra-day and inter-day precision respectively. It was found that RSD% values on average for both molecules are appropriate to get precise results within the method. Based on signal-to-noise rate (S/N) of 3 and 10, the limits of detection (LOD) and quantification (LOQ) were determined by UHPLC-MS/MS using the standard solutions of target compounds. AFGP and sorbic acid were easily detected due to good S/N and LOQ levels. The calculated LOD levels were 27.6 µg/kg and 49.1 µg/kg for sorbic acid and AFGP respectively. The resulting LOQ values were 32.0 µg/kg and 56.5 µg/kg for sorbic acid and AFGP respectively.

3.5. Application to real samples

For preliminary specificity study, honey samples were adulterated deliberately with both BRS and WRS separately at the concentration levels of 10%, 20%, and 40% (w/w). They were analyzed using the developed method and the linear responses with high specificity were found for AFGP and sorbic acid respectively. The developed methodology enabled a rapid, highly specific, and sensitive detection of honey adulterated with 10% or less rice syrup from different origins. The obtained chromatograms of the mimicked samples of adulterated honey are given in Fig. 5 (Fig. 5 near here).

In this study, 2 BRS, 1 WRS, 3 beet syrups, 2 HFCS samples, and 107 honey samples were analyzed. Arsenic analysis was also performed quantitatively at ICP-MS to confirm positive results. As predicted, AFGP was found in WRS in an apparent amount but the absence of sorbic acid was observed in WRS. Other syrup samples from different origins were reported as negative in terms of both sorbic acid and AFGP concentration. Thereby these markers could be depicted as unique to rice syrups. According to the investigation of the presence of AFGP and sorbic acid in BRS, a high amount of sorbic acid was detected in one of the BRS samples, while the presence of AFGP was not observed. In the other BRS sample, a low amount of AFGP could be quantified simultaneously with sorbic acid. These results revealed that AFGP is a good choice of marker for WRS but not for BRS and a novel marker should be validated in order to identify the adulteration of honey with BRS. Representative chromatograms of the adulteration identified samples are given in Fig. 6 (Fig. 6 near here).

At WRS, arsenic was not quantified. On the vice versa, supporting the literature, high levels of arsenic were detected in BRS samples. 75.9 µg/kg and 56.2 µg/kg arsenic residues were detected at two BRS samples. Comparable values for BRS samples were reported previously (Jackson

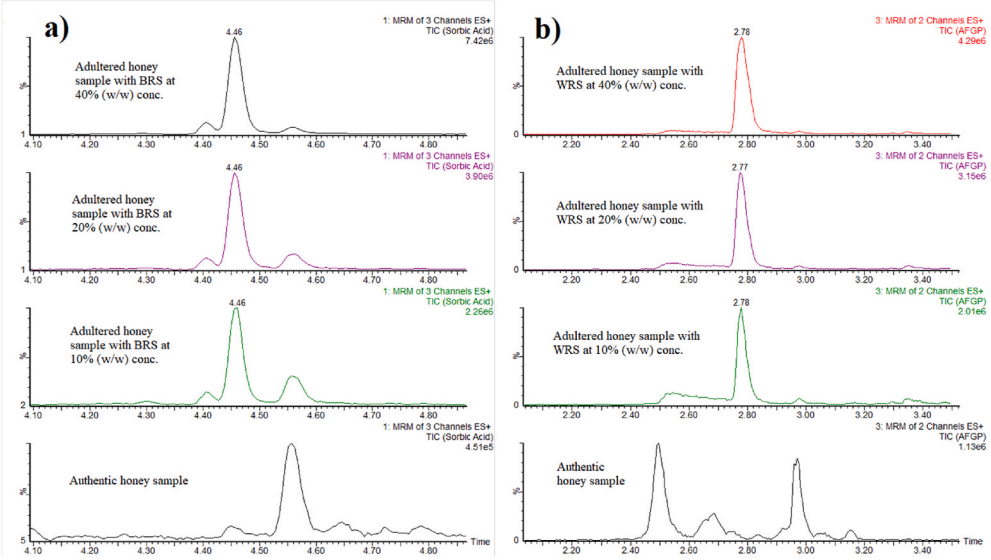

Fig. 5. Resulting sorbic acid and AFGP responses regarding the authentic honey sample, BRS and WRS added samples at the concentrations of 10%, 20%, and 40% (w/w); a) TICs of the sorbic acid responses at authentic and adultered honey samples, b) TICs of the AFGP responses at authentic and adultered honey samples.

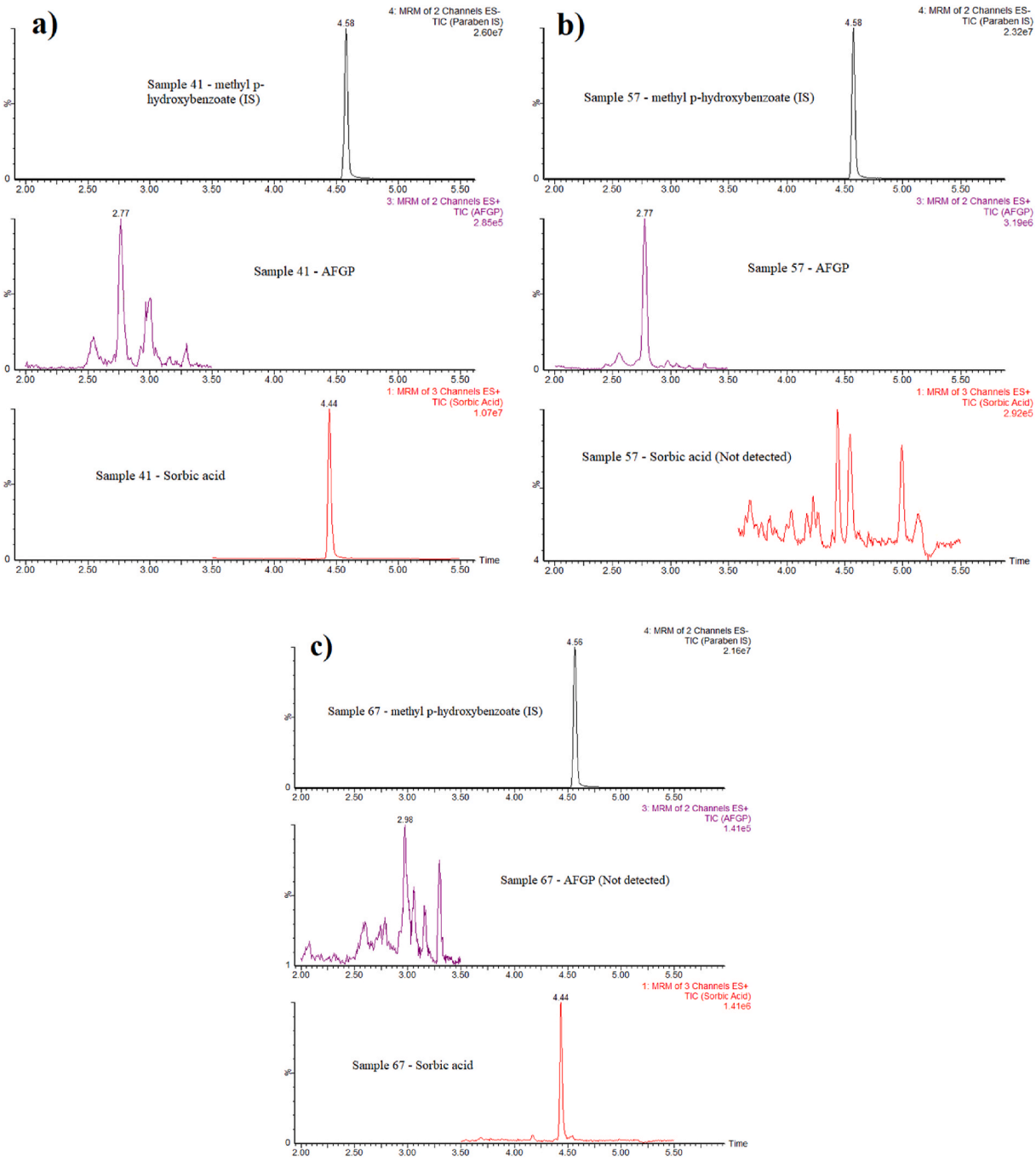

Fig. 6. TICs of the BRS and WRS detected samples. a) Honey sample in which co-occurrence of both markers, b) Honey sample with the presence of WRS and resulting AFGP signal, c) Honey sample with the presence of BRS and resulting sorbic acid signal.

et al., 2012). Furthermore, authentic chestnut honey, HFCS, and beet syrup were also containing a conspicuous amount of arsenic residue. In honey samples, only 2 of the samples in which rice syrup was detected had residues with values of 20.5 μg/kg and 40.2 μg/kg. At this point, it could be said that there is no correlation between the presence of rice syrup and arsenic values when the sample type is adultered honey. The dilution of BRS after addition to honey and the corresponding arsenic values may cause these uncorrelated results. In our opinion, arsenic residue analysis alone cannot be used as a confirmatory analysis and it is not a unique and reliable marker for rice syrups determinations in honey. Honey samples were also analyzed to insight the general adulteration prevalence made with rice syrups. 24 out of 107 samples were identified as adultered by adding rice syrup. 3 of them were considered to be adultered by adding WRS due to observation of only AFGP residue. The remaining 21 samples were adultered by adding BRS since the sorbic acid residues were detected alone or together with AFGP. In 6 samples, sorbic acid and AFGP markers were present together. These samples were reported to be adulterated with BRS, since sorbic acid was considered as a marker molecule unique to BRS in our study, and AFGP concentrations could be detected in some BRS other than sorbic acid. Only the presence of sorbic acid was detected in 15 samples. We have found 2.15 mg/kg and 3.82 mg/kg sorbic acid contents in BRS-1 and BRS-2 respectively. An average of 0.60 mg/kg sorbic acid content was quantified in 21 honey samples found to be adulterated by BRS, and the sorbic acid content of the samples ranged from 0.08 mg/kg to 1.25 mg/kg. In the study conducted by Xue et al. (2013), 186 honey samples

were analyzed and an average of 108.4 mg/kg AFGP was reported within the concentration range from 21.5 to 145.6 mg/kg in 16 samples. The average of AFGP quantified in 9 honey samples out of 107 samples analyzed within the scope of our study was determined as 0.53 and the calculated AFGP levels of the samples ranged from 0.10 mg/kg to 1.66 mg/kg. These results showed us that adulteration in honey using rice syrup is mainly done with BRS instead of WRS. The color similarity to honey and the detectability of WRS by food control laboratories can be shown as hypotheses explaining the preference of BRS over WRS. In this respect, it can be mentioned that many adulterated honey samples may be overlooked by only analyzing the AFGP marker. Table 2 summarizes the results of syrup samples and honey samples in which merely rice syrup was detected. The results of all 107 samples are shared in Table 1S (Table 2 near here).

As can be seen from the quantitative results in Table 2., AFGP and sorbic acid residues can be detected in highly variable amounts. We do not find it very logical to quantitatively report the markers because AFGP or sorbic acid residue can be seen in different amounts in rice syrups. In this respect, even if the syrup is mixed with honey at the same percentage, it may be possible to detect variable amounts of sorbic acid or AFGP. This situation prevents understanding how much rice syrup is added as a percentage. Since we did not detect the content of AFGP and sorbic acid in natural honey, we evaluated any signal of these markers which above the determined LOQ of the method as positive and we found it more conceivable to give a qualitative analysis result. BRS is a type of sweetener that might be produced by fermenting brown rice with certain enzymes to disintegrate the starch content. Then the fermented liquid is strained and cooked until it becomes syrup. The use of processes like fermentation and germination has been established as an efficient method for releasing bound bioactive compounds such as organic acids, phenolics, fatty acids (Tyagi et al., 2021). Products made from cultured brown rice can replace typically used chemical preservatives such as potassium sorbate, calcium propionate, and benzoates to provide a consumer-friendly, and allergen-free shelf-life extender alternative (Saleh, Wang, Wang, Yang, & Xiao, 2019). Brown rice germination and/or further fermentation process might be included in BRS production and it may result in high sorbic acid content in BRS. The potential high content of the short-chain fatty acid composition of brown rice has led us to conclude that it would probably be unreasonable to preserve BRS by adding any additional chemicals such as sorbate. In this respect, it is quite logical that a high amount of sorbic acid is detected in RS produced from brown rice and that sorbic acid residues are detected in honey samples adulterated with these syrups. Astoundingly, during our research, we determined that BRS can also be added to honey for color mimicking. In some samples, apparent C-4% levels and sorbic acid was also quantified simultaneously. This case was considered as that the corn syrup has a different shade of yellow than honey and in order to simulate this color to honey color, it was interpreted as BRS was added to honey,

Table 2
Analysis results of sugar syrups and authentic honey samples of different origins together with the samples in which rice syrup addition was detected from analyzed 107 honey samples.

Sample ID	AFGP (WRS marker), (±)/(conc. mg/kg)	Sorbic acid (BRS marker), (±)/(conc. mg/kg)	[a]Arsenic residue conc. (μg/kg)	Estimated origin of the rice syrup	Adulteration assessment (Pass/Fail)
BRS-1	(+)/(2.51)	(+)/(2.15)	75.9	B	NA
BRS-2	(−)	(+)/(3.82)	56.2	B	NA
WRS-1	(+)/(46.81)	(−)	ND	W	NA
Beet syrup −1	(−)	(−)	23.1	NA	NA
Beet syrup −2	(−)	(−)	52.5	NA	NA
Beet syrup −3	(−)	(−)	ND	NA	NA
HFCS -1	(−)	(−)	18.2	NA	NA
HFCS -2	(−)	(−)	ND	NA	NA
[b]Authentic Chestnut Honey (4)	(−)	(−)	74.5	NA	Pass
[b]Authentic Blossom Honey (10)	(−)	(−)	ND	NA	Pass
[b]Authentic Pine Honey (6)	(−)	(−)	ND	NA	Pass
13	(−)	(+)/(0.59)	ND	B	Fail
16	(−)	(+)/(0.49)	ND	B	Fail
24	(−)	(+)/(0.26)	ND	B	Fail
25	(−)	(+)/(0.90)	ND	B	Fail
35	(+)/(0.23)	(+)/(0.72)	ND	B	Fail
40	(−)	(+)/(0.65)	ND	B	Fail
41	(+)/(0.20)	(+)/(0.89)	ND	B	Fail
42	(+)/(0.31)	(+)/(1.04)	21.5	B	Fail
43	(−)	(+)/(0.21)	ND	B	Fail
48	(−)	(+)/(0.13)	ND	B	Fail
49	(+)/(0.50)	(−)	ND	W	Fail
57	(+)/(1.66)	(−)	40.2	W	Fail
58	(−)	(+)/(0.82)	ND	B	Fail
65	(−)	(+)/(0.08)	ND	B	Fail
67	(−)	(+)/(0.75)	ND	B	Fail
68	(−)	(+)/(0.55)	ND	B	Fail
73	(−)	(+)/(0.21)	ND	B	Fail
80	(−)	(+)/(0.64)	ND	B	Fail
82	(−)	(+)/(0.67)	ND	B	Fail
84	(−)	(+)/(0.13)	ND	B	Fail
87	(+)/(0.44)	(+)/(1.25)	ND	B	Fail
88	(+)/(0.11)	(+)/(0.74)	ND	B	Fail
89	(+)/(1.30)	(−)	ND	W	Fail
106	(+)/(0.10)	(+)/(0.92)	ND	B	Fail

BRS; Brown rice syrup, WRS; White rice syrup, HFCS; High fructose corn syrup, ND; Not detected - Quantified under LOQ level (10 μg/kg) of arsenic analysis method, NA; Not applicable, W; White rice syup, B; Brown rice syrup, (±); The presence or absence of marker compounds.

[a] Arsenic analysis were only performed in the case of the occurance of sorbic acid and/or AFGP positivenes.

[b] Numbers in parentheses for sample ID indicate the number of samples studied.

besides corn syrup. Starch analysis on rice syrup marker positive samples was also performed for confirmation. The results displayed that greater than 80% of the samples were in correlation with the presence of starch residue. Since the starch residue in honey may differ depending on the type of rice syrup used and the amount added to the honey, in our point of view that the correlation above 80% is an affirmative rate to confirm the results. In summary, honey samples adultered with BRS in the market are difficult to detect using the available and traditional methods. Thanks to our new method, the findings pinpointed that sorbic acid meets the requirements of a convenient adulteration marker.

4. Conclusions

We believe that sorbic acid could be either a naturally occurring compound or a kind of by-product which could be exposed during the BRS production process. For the proof of the concept and in order to understand more clearly whether this substance is a natural component or process related molecule, further research can be done using more and different BRS. The developed method is promising in terms of reliable detection of honey authenticity and it was also thought to be capable to determine the adulterant origin since the markers can be quantified separately for the BRS and WRS. Moreover, it was understood that arsenic residue analysis alone is not a confident assay for detecting the presence of rice syrup. By performing the developed practical, cost, and time effective method, 24 out of 107 analyzed honey samples were found to be adulterated with rice syrup. 21 of them were considered to be adultered by adding BRS and the remaining 3 samples were adultered by adding WRS. According to the data obtained, it was seen that adulteration was mostly made with BRS instead of WRS and that adulteration may be overlooked with existing methods due to the BRS positiveness could not be confirmed by the AFGP and arsenic results. The reported adulteration frequency within this study underlined that honey adulteration and counterfeiting using rice syrups is a critical issue and needs to be handled carefully. We suggest adding sorbic acid into the routinely used AFGP analysis methods since BRS may include trace levels of AFGP, and honey can also be adultered by this origin syrups. Consequently, we envisage that at analytical method acquisition, integration of the sorbic acid monitoring along with AFGP identification, thereby updating the current rice syrup detection methods may promisingly secure the reliable assessment of honey adulteration when the rice syrup was preferred to use as an adulterant.

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CRediT authorship contribution statement

İsmail Emir Akyıldız: Writing – original draft, preparation, Methodology, Conceptualization. **Dilek Uzunöner:** Methodology. **Sinem Raday:** Writing – review & editing. **Sezer Acar:** Investigation, Conceptualization. **Özge Erdem:** Resources, Investigation. **Emel Damarlı:** Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112618.

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