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Straightforward monitoring of honey with foreign diastase by leveraging the differentiation in LC-UV proteome profiles of authentic and fraudulent samples

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ABSTRACT

Honey adulteration using sugar syrups is ubiquitous and adulterated honey may also be prepared by direct addition of α-amylases from different sources to match the legislation for honey trade. Colorimetric diastase assays may report false negatives in terms of foreign diastase (FD) detection owing to using substrates being prone to be cleaved by any α-amylases. The main objective of this study was to develop a reliable analytical method to determine FD contained honey. For this purpose, intrinsic honey proteins were first isolated, enriched, and cleaned up via experimentally designed serial ultrafiltration methodology. Next, diastase and invertase as abundant honey enzymes were purified simultaneously from obtained crude protein isolate by means of FPLC and novel purification protocol. Total protein and enzyme activity assays along with SDS-PAGE analysis were conducted for purity check. Subsequently, two purity-confirmed enzymes were used as analytical references in method development. With the hyphenation of UV detection, optimized chromatographic resolution, and practical dilute & shoot sample pretreatment, Foreign Amylase Monitoring (FAM) method has been introduced for routine analysis. Purified authentic enzymes were labeled in LC-UV chromatogram of honey proteome profile to distinguish any unnatural enzyme/protein signal. According to the validation of the FAM method, precise (RSD_R; 1.27%), and linear (mean R^2 >0.995) results were able to obtain. Commercial honey samples ($n = 202$) collected over 3 consecutive years were probed using the FAM method and industrial α-amylases were also analyzed for verification. The diastase activities were also measured prior to FAM application to elucidate if any positive correlation. The developed method rapidly and reliably detected the presence of FD qualitatively. A total of 74 samples were found to contain FD. It was observed that the presence of FD was correlated in samples with abnormally high diastase activities and an apparent FD peak was identified readily in all enzyme adulterated samples. The developed FAM method and performed trend analysis allowed us to decipher a dramatic increase in FD existence and this revealed the recently emerging problem of honey authenticity.

1. Introduction

Honey is one of the most essential and functional foodstuffs due to its rich taste and nutritional value as well as its endowed health benefits and therapeutic properties [14]. The amount of protein in honey is much less than 0.1–0.5% of honey dry matter [22,32]. The origin of the

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proteoforms found in honey is attributed to the honeybee's hypopharyngeal glands (HPG). The rest of the proteins are of pollen and vegetal origin. In general, about 7 to 19 protein bands have been reported in natural honey [21]. Excluding pollen and plant proteins, the predominant type of encoded proteins found in honey are the enzymes and major royal jelly proteins (MRJPs) [37]. Honey contains several enzymes in varying concentrations depending on its botanical origin. The most important of these are discernable levels of diastase (α-amylase) and invertase (α-glucosidase) originating from HPG and salivary gland secretions [4]. α-glucosidase and α-amylase act a crucial role in honey ripening and their activities are criticized as reliable indicators of honey quality and freshness [27]. The most negatively affected biochemical components of unprocessed honey after careless processes such as improper heating or extended shelf life are diastase and invertase enzymes [28]. Dilution of honey by the addition of sugar syrups may also result in low enzymatic activities. Hence, these abundant enzymes' total activities have been assumed as a critical quality parameter for honey trade [19]. The European Commission (EC) adopted the Codex Alimentarius Standards for honey [12] and, to establish quality criteria for honey, issued the directive 2001/110/EC which was amended by 2014/63/EU [13]. One of the key quality parameters in these guidelines is diastase activity expressed as the diastase number (DN). According to the Schade scaling, the total diastase activity of the honey must not be less than 8 DN and not be less than 3 DN for some types of honey with low natural enzyme content (e.g. citrus honey).

The DN of honey is measured colorimetrically using isolated starches, dye-linked starches, or p-nitrophenyl conjugated specially synthesized short-chain oligosaccharides as substrates. Official analysis methods for determining diastase activity in honey are Schade [29] and Phadebas® [11] assays as recommended by International Honey Commission (IHC). Schade procedure has also been included in the standard methods of honey analysis in the Codex Alimentarius [12]. In addition to the abovementioned standard methods, diastase activity can be measured by using commercial kit (Ceralpha method, Amylazyme tablets) with an identical methodological basis developed by Megazyme company [24]. Alternatively, in the literature, amylase activity in honey has been measured spectrophotometrically after the stepwise hydrolysis of α-(1–4)-glucosidic bonds in 4,6-ethylidene-p-nitrophenyl-α-D-maltoheptaoside [7].

The high price of honey and the growing demand direct the manufacturers or beekeepers to make adulteration and fraud-oriented practices in order to gain more economic profit. It is emphasized that no additives will be accepted in honey according to Codex Alimentarius and European Union Commission criteria [12]. Inexpensive sweeteners such as high fructose corn syrups (HFCS), rice syrups, and inverted cane or beet syrups are ubiquitously used in honey adulteration [5,18]. Identification of the counterfeit honey has attracted great attention and to address this issue, a great deal of analytical approaches have been developed. As the targeted techniques covered in literature; 3-acetyl furan glycopyranoside (AFGP), sorbic acid, and arsenic marker determination for rice syrup addition [2], C4% (SCIRA) analysis for cane or corn syrup identification [30], detection of difructose anhydride [25], and oligosaccharide screening [6] are the common adulteration analysis methods. Furthermore, there are several spectroscopic and mass-spectrometric non-targeted-based, chemometric modalities including infrared [17], nuclear magnetic resonance (NMR) [10], fluorescence spectroscopy [15], and high resolution mass spectrometry (HRMS) based metabolomic/foodomic methods [16,23,38].

The criteria set for diastase levels bring with it a new and growing concern about authenticity. Recently, the spectrum of honey adulteration includes new approaches such as adding α-amylases derived from disparate origins to match the legislation. Foreign diastase (FD) can be added to honey in order to mask the descending natural diastase values due to heat treatment or lengthy storage or to artificially adjust the natural diastase values in honey diluted as a result of the addition of tailor-made sugar syrups. FD can also be added to digest the starch residues that may arise from the syrup in honey [33]. There are enzymatic assays to determine the foreign enzymes occurrence arising from the syrup addition such as foreign invertase (β-fructofuranosidase, BFF) analysis to detect inverted beet syrup or β/γ-amylase assay for identifying the starch-based syrup addition [35,35]. Calculating the total activities of the authentic enzymes allows us to verify the quality and purity of honey. Nonetheless, activity assays are not specific in terms of honeybee's α-amylase owing to used substrates being prone to be cleaved by any amylases which hydrolyze α-1,4 glycosidic bonds. Therefore, colorimetric procedures may report false negatives in the case of spurious origin α-amylase (have not been expressed by *Apis mellifera* L.) availability.

Recently, a label-free untargeted proteomic technique was discussed in the literature to elucidate the FD issue in honey [16]. Even if this approach is promising and versatile at both adulteration identification and FD origin determination, high instrumental cost, complicated data annotation and processing, tedious and laborious micro-proteomic handling steps seem primary bottlenecks for implementation of the method in routine workflows. Moreover, the usage of targeted proteomics instead of discovery mode proteomics by constructing Selected Reaction Monitoring acquisition libraries by forming signature quantotypic peptides for α-amylases from various organisms is a challenging task and labor-intensive work to deal with. Additionally, this approach can be mentioned as an error-prone methodology that may give false-negative results that is performed with α-amylases obtained from different organisms except for the targeted ones. Therefore, it is essential to present a superior untargeted/profiling method in terms of cost and practicality to unveil whether the noted diastase activity originated from bee-derived α-amylase or was caused by latent adulteration.

Several advanced analytical techniques may be evaluated to identify FD. Accustomed gel-based proteomic techniques like SDS-PAGE, 2D electrophoresis, or isoelectric focusing are inefficient in identifying FDs since MWs and isoelectric points (pI) of the bee diastase and amylases from other sources are very close. Likewise, as another separation technique based on MW fractionation, SEC also cannot separate the FDs from natural diastase. On the other hand, activity-based methods are leaned to give false negatives even if strict reaction pH and incubation conditions were established for each FD. The presence of FD and its hydrolytic affinity against chosen substrates can interfere with the bee diastase activity and thereby the artificially up-regulated DN could not be reported. It is also arduous to develop new enzymatic activity assays for each FD and they may also generate misinterpretations in case of the usage of new/different FDs. Besides, it is impractical and expensive to raise primary antibodies for each FD and to develop an immunoassay-based methodology. Hence, techniques that present greater resolution such as protein liquid chromatography are more amenable and powerful tool to distinguishing the FD in honey. Various bind & elute mechanisms could be considered under the LC method development like IEX or capillary electrophoresis, hydrophobic interaction chromatography, or affinity chromatography (Lectin or Concanavalin A) leveraging the varying glycoprotein structures of the honey proteoforms. In the present study, we aimed to separate and identify the FDs by using the LC-UV technique combined with reversed-phase (RP) protein chromatography and dilute & shoot sample pretreatment. With this novel profiling methodology, any discriminant FD response reflects the manipulation whether arising by starch-based syrups addition or direct enzyme addition. Therefore it was indispensable to screen FD in honey with high specificity and improved resolution. We hypothesized that, to successfully differentiate the LC-UV chromatogram and spectra profile of the adulterated honey we had to tag authentic honey enzymes first. Thereby, efficient purification of diastase and invertase was a critical step for FAM development. Currently, there are no protocols reported for the simultaneous, effective and practical purification of invertase and diastase originating from *Apis mellifera* L. in honey. Albeit there are few publications on the isolation and purification of α-glucosidase and α-amylase in honey and in HPGs of *Apis mellifera* L., these studies are not

easy to transfer and used methodologies are outdated and time consuming [7,8,26]. In our study, we also dedicated ourselves to contributing to the literature by developing a novel, simultaneous tandem purification methodology for honey enzymes. For this, the abundant honey enzymes obtained as crude extract were subjected to successive orthogonal purification.

Our primary focus of interest was to be able to report FD contained honey in an accurate, straightforward, and cost-effective way. Herein, actual work was designed to detect FD containing adulteration variants by harnessing the Liquid Chromatography (LC-UV) proteome profiling and to compare the protein distributions of genuine and fraudulent honeys.

2. Method and materials

2.1. Reagents and chemicals

Sodium chloride (NaCl), trifluoroacetic acid (TFA, LC grade), Trizma® base, and acetonitrile (ACN, LC grade) were supplied from EMD Chemicals Inc. (Gibbstown, NJ, U.S.A.). Calcium chloride ($CaCl_2$), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and sodium maleate were purchased from Sigma-Aldrich® (St. Louis, MO, U.S.A.). The tris base (≥99.8%) and beta-mercaptoethanol (BME, ≥ 98%) (14.2 M) were purchased from Bio-Rad®, Inc. (Hercules, CA, U.S.A.).

2.2. Sample collection

Authentic honey samples from different botanical origins produced under stringent conditions were collected directly from beekeepers and used in method development studies. From divergent provinces in Türkiye, a total number of 20 honey samples (citrus $n = 5$, chestnut $n = 5$, pine $n = 5$, blossom $n = 5$) were supplied in 2021. All cohorts were subjected to authenticity, physicochemical, and palynological tests before use. In physicochemical tests; moisture, 5-hydroxymethyl furfural content, acidity, conductivity, fructose/glucose ratio, total percentage of disaccharides and oligosaccharides [11], total diastase, and invertase activities were measured. For the detection of any adulteration, BFF residue [36], β/γ-amylase residues, rice syrup markers (AFGP & sorbic acid) [2], and the presence of C4 sugars (SCIRA) [1] were examined in the candidate samples. In order to evaluate the potential protein profile variations in the FAM method, samples were palynologically verified and classified. Palynological test of authentic honeys was performed by slightly modifying the previously published method [34]. Authentic pine honey was elected as the method development material due to its rich proteinaceous content. Analysis results of the selected pine honey are given in Table S1. Other authentic honey samples were assessed only in validation. Apart from these, 202 honey samples were randomly gathered from the market in 2020 ($n = 30$), in 2021 ($n = 124$), and in 2022 ($n = 48$) years for comprehensive real sample analysis. The applicability of the developed FAM method was evaluated using these commercially available samples. Trend analysis was also aimed to elicit the initial point of the questioned conundrum by sampling for three consecutive years. Prior to the FAM method application, diastase activities were also measured to see if there is any activity-dependent correlation. All honey samples were taken into glass jars, hermetically sealed, and stored at 4 °C temperature, in an inert environment.

Industrial α-amylases from different sources were also purchased and the FAM method's ability to identify the presence of FD variants was investigated. We supplied the powder form of *Aspergillus oryzae* α-amylase from Sigma-Aldrich® (St. Louis, MO, U.S.A.) and tested as an FD alternative. As a bacterial source and heat-stable form of α-amylase, *Bacillus licheniformis* was supplied from Sigma-Aldrich®. For expanded verification, barley α-amylase from barley malt (*Hordeum vulgare* L., A2771), Sigma-Aldrich® was also inserted in the study. Two different Alphamalt-A15140 enzyme formulations as commercially available FD (*Aspergillus oryzae*) products were also purchased and used for the preparation of the FD fortified mimicked samples.

2.3. Extraction & enrichment of total honey proteome

We followed the procedure in our previously published research to efficiently extract the total honey proteome [3]. In this study, two different ultrafiltration (UF) equipments were used sequentially for the sake of extracting and concentrating the honey enzymes, as well as eliminating co-existing small molecules of pine honey. Amicon® stirred cell UF apparatus equipped with Biomax® 10 kDa PES (polyethersulfone) ultrafiltration membrane (Merck, Darmstadt, Germany) was primarily used to concentrate high volume filtrated and pollen depleted pine honey solutions prepared in 50 g/400 mL concentration. The latter step UF took place to further concentration, passive diafiltration, and buffer exchange purposes using a Centrifugal Ultrafiltration Unit (CFU) apparatus (Amicon® Ultra-15, 10 kDa MWCO, 15 mL).

2.4. Purification of diastase and invertase

All purification processes were completed at Fast Protein Liquid Chromatography (FPLC) system (NGS Quest 10, Bio-Rad, Hercules, CA, U.S.A.) equipped with a fraction collector, conductivity, and Ultraviolet detector. To ensure enzyme stabilization over the course of purification, cold condition (4 °C) was established by placing the FPLC and fraction collector into a refrigerator. ChromLab software was used for data evaluation. Track of the purification performance for each step was accomplished by using enzyme activity assays, total protein assay, and gel electrophoresis.

At the first step of the protein fractionation, approximately 1 mL of crude protein extract obtained from the UF process was submitted to mix-mode chromatography using a prepacked multimodal column (20×1.0 cm, Foresight™ CHT™ XT, 40 μm, packed with ceramic hydroxyapatite (CHT), (Bio-Rad, Hercules, CA, U.S.A.). The CHT column was equilibrated with binding buffer (5 mM phosphate buffer at pH 6.6) and then proteins were eluted at a flow rate of 1 mL/min by employing a linear 5 to 400 mM gradient of [PO_4^{3-}] in elution buffer (50 mM Phospate-buffered saline (PBS), pH 7.0). The pH was kept constant along the binary gradient and the elution of enzymes was achieved by applying a phosphate gradient. The run time was 35 min. Both absorbed and flow-through (unbound) proteins were collected from the column by employing the gradient program as follows; 0.00–5.00 min; 100% mobile phase A (MP-A) – 0% mobile phase B (MP-B) (equilibration), 5.00–6.00 min; 100% MP-A – 0% MP-B (loading), 6.00–11.00 min; 100% MP-A – 0% MP-B (removal of the unbound molecules), 11.00–26.00 min; 0% to 70% MP-B (1st elution), 26.00–31.00 min; 70% to 100% MP-B (2nd elution), 31.00–35.00 min; 100% MP-A – %0 MP-B (regeneration). Each fraction from the CHT purification step containing either diastase activity or invertase activity confirmed was collected in a volume of 1.0 mL, pooled, and subjected to the next step of purification utilizing the anion exchange (AEX) chromatography. Approximately 1 mL of the pooled fractions of diastase and invertase was injected individually into an AEX column (Enrich™ Q, 5×50 mm, 10 μm, 1 mL, BioRad, Hercules-California, U.S.A.) and target enzymes were liberated from the resin by employing a linearly increased salt gradient. Mobile phase contents used; for MP-A; 10 mM NaCl, 10 mM Tris.HCl Buffer pH 7.4 and MP-B; 1 M NaCl, 10 mM Tris.HCl Buffer pH 7.4. The flow rate was 0.65 mL/min and the run time was 40 min. The following gradient program was used; 0.00–5.00 min; 100% MP-A – 0% MP-B (equilibration), 5.00–6.54 min; 100% MP-A – 0% MP-B (loading & binding), 6.54–11.54 min; 100% MP-A – 0% MP-B (intermediate purification/washing), 11.54–26.54 min; 0% to 60% MP-B (elution-1), 26.54–31.54 min; 60% to 100% MP-B (elution-2), 31.54–40.00 min; 100% MP-A – 0% MP-B (regeneration). The fractions containing diastase and invertase activity were collected in volumes of 1.0 mL, pooled again, and

quantitatively transferred to the final step of purification. The pooled fractions derived from AEX purification were further subjected to size exclusion chromatography (SEC) individually without applying any pre-concentration. Consequently, polishing step was carried out meticulously for each enzyme and for this, ENrich™ SEC 650 column (10×300 mm, 10 μm, MWCO; 5 kDa-650 kDa), (BioRad, Hercules-California, U.S.A.) was pre-activated. Enzymes were isocratically eluted using the same buffer containing 10 mM Tris.HCl (pH 7.4) and 100 mM NaCl at a flow rate of 0.5 mL/min. The sample injection volume was 250 μL and the run time was 60 min. The fractions of interest were collected in the volume of 500 μL and then stored at 4 °C. The purity of the collected samples and their ability to form an analytical reference for the LC-UV method was tested by using gel electrophoresis described in Section 2.6. Purification protocol is depicted briefly in Fig. 1.

2.5. Determination of the purification performance

The performance of the purification procedure was tracked both in the final and intermediate steps for each enzyme by calculating the total protein concentration (mg/mL), total protein amount (mg), total enzyme activities (Schade Unit/g = DN for diastase and U/kg for invertase), specific activities (U/mg protein), yield/recovery (%), and purification fold/factor.

2.6. SDS-PAGE analysis

The diluted (20-fold) crude protein extract of pine honey along with the samples collected at the end of the each purification steps were subjected to SDS-PAGE analysis and proteins were separated electrophoretically in Mini-PROTEAN® Tetra Cell (Bio-Rad® Inc., Hercules, CA, U.S.A.) using 4–20% Mini-PROTEAN® TGX™ Precast/stain-free gels. The tris/glycine buffer was employed as running buffer. 60 μL from each sample was mixed with 20 μL of sample buffer that contains 10% of BME as reducing agent and 90% of 4X Laemmli buffer. Samples were completely reduced and denatured in heat block for 10 min at 70 °C. 45 μL aliquots were loaded to gels along with an unstained protein ladder (Precision Plus Protein™, Bio-Rad®). Band visualization was accomplished at Gel Doc™ EZ Imager system using Image Lab software.

2.7. Total protein assay

Total protein assay was conducted by means of the Quick Start™ Bradford protein assay kit (Bio-Rad®, Inc., Hercules, CA, U.S.A.). Samples (20 μL) were taken into spectrophotometer cuvettes and mixed with 1 mL of dye Reagent (Coomassie stain G-250). The absorbances of the samples were read at 595 nm. The calibration plot was constructed using Bovine Serum Albumin standards with 7 points (2.0, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125 mg/mL). Thermo Scientific™ Evolution™ 350 UV–Vis Spectrophotometer (Waltham, Massachusetts, U.S.A.) was operated, and all measurements were recorded against reagent blank.

2.8. Enzymatic activity assays

2.8.1. Determination of the invertase activity

Invertase activity measurements were performed with minor modifications of the standard IHC-2009 method. The fractions derived from each purification steps were analyzed directly without any buffer dilution. For this, 2.5 mL solution of pNPG (0.02 M pNPG, CalbiochemR, CA, U.S.A.) in 0.1 M pH 6.0 phosphate buffer was incubated in a water bath at 40 °C for 5 min. Then 0.25 mL of the sample solution was added and mixed well. The resulting mixture was placed in a water bath and incubated for 20 min at 40 °C. Subsequently, 0.25 mL of 3 M trizma base, pH 9.5 as stopping reagent was added and test tube was vortexed thoroughly for quenching. Absorbances of the samples were read on UV–Vis spectrophotometer at 400 nm. Total invertase activity was calculated according to following Eq. (1);

$$Totalinvertaseactivity(U/kg) : 158.94 \cdot \Delta Abs^{*} \quad (1)$$

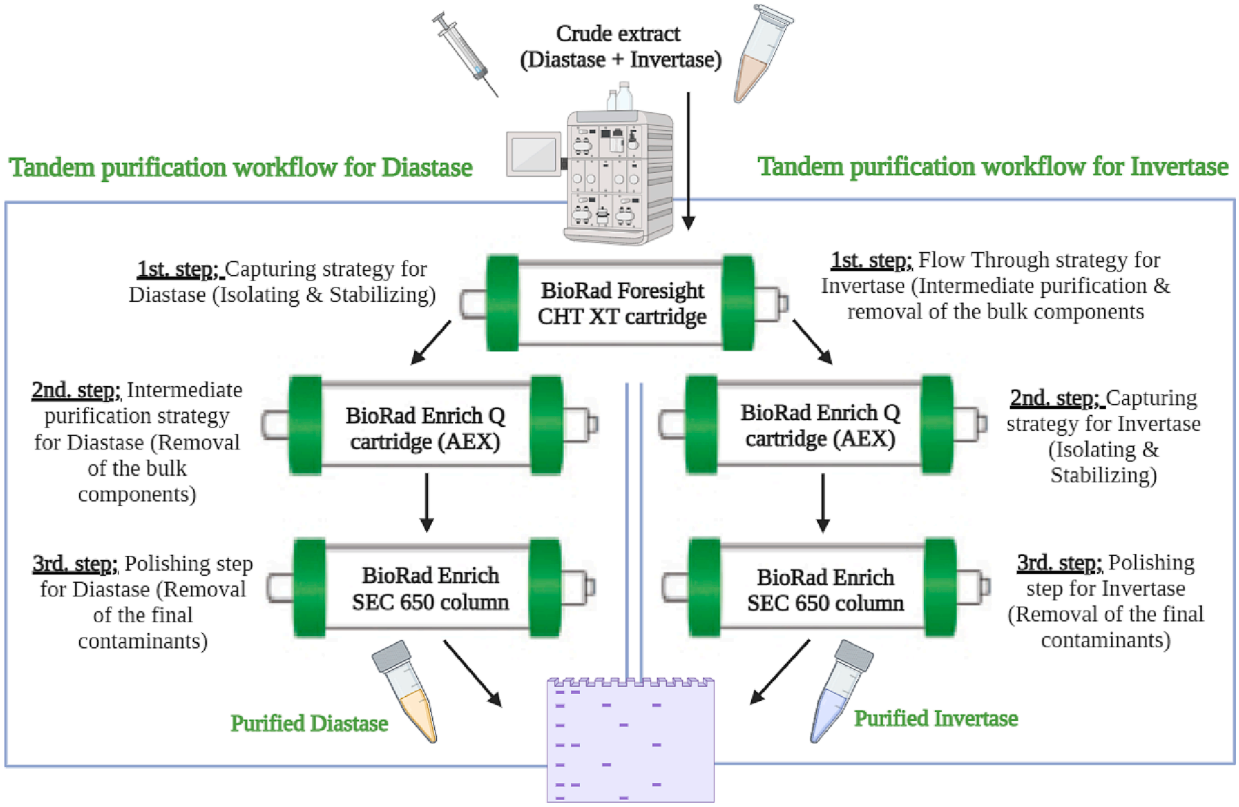

Fig. 1. Schematic overview of the three steps tandem purification workflow.

*ΔAbs; Absorbance differences between reagent blank and sample.

2.8.2. Determination of the diastase activity

In order to observe the diastase activity, slightly modified assay kit (T-AMZHY, Megazyme, Wicklow, Ireland) was adapted. The Amylazyme® tablets supplied as substrate in the kit are similar to the Phadebas® tablets but contain cross-linked dye-conjugated amylose instead. 1 g of honey was thoroughly homogenized using 20 mL of 100 mM sodium maleate buffer (pH 5.6) and completed to 25 mL. Resulting solution (1 mL) was transferred to 15 mL centrifuge tube and the sample was incubated in a water bath at 40 °C for 5 min. An Amylazyme® tablet has added and the tube was incubated at 40 °C for 10 min. 10 mL of trizma base solution (2% (w/v)) was added to terminate the reaction. Afterward, the sample was spun at 4000× *g* for 5 min and the supernatant was transferred into the spectrophotometer cuvette. The absorbance of the sample solutions was then measured against sample blank using at 590 nm. Results in Schade units were calculated by the following formulation (2);

$$Amylaseactivity(DN);(SchadeUnit/1gofhoney) = 20.0.\Delta Ab \quad (2)$$

For the fractions derived from the protein purification, 1 mL of the representing liquid was transferred into 15 mL test tube and diluted 1:2 with the 2 mL of 100 mM sodium maleate buffer (pH 5.6) to adjust the reaction pH. 1 mL aliquot of this sample was subjected to assay protocol as defined above. DN value was multiplied by 3 as the dilution factor.

2.9. FAM method conditions

2.9.1. Sample pretreatment

The straightforward dilute & shoot protocol was optimized as sample preparation strategy. For this, 2.5 g of honey sample was weighed and 7 mL of PBS (pH 7.4) was added into a 15 mL centrifuge tube. Sample was agitated until homogenization. The resulting solutions were filtered into glass vials using a 0.45 μm PVDF syringe filter and injected into the LC system.

2.9.2. Instrumental conditions

LC-UV analysis of the FD was conducted using the Waters® Alliance HPLC system (Milford, Massachusetts, U.S.A.) equipped with UV detector. Invertase, diastase, FD, and other honey proteins were resolved by applying a shallow gradient elution using MP-A as 5% ACN, 95% USS, 0.1% TFA, and MP-B as 5% USS, 95% ACN, 0.1% TFA at a flow rate of 0.7 mL/min. Injection volume was 20 μL, column temperature was maintained at 30°C, and the autosampler was held at 4 °C. The run time was 30 min and binary gradient was as follows; 0.00–1.00 min; 95% MP-A – 5% MP-B, 1.00–15.00 min; 5% to 60% MP-B, 15.00–20.00 min; 60% to 85% MP-B, 20.00–21.00 min; 15% MP-A – 85% MP-B, 21.00–22.00 min; 85% to 5% MP-B, 22.00 – 30.00 min; 95% MP-A – 5% MP-B. Fixed UV wavelength was set as 280 nm. A superficially porous (SP) column, BioResolve RP mAb polyphenol, 150×4.6 mm i.d, 2.7 μm (Waters®, Milford, MA, U.S.A.) was used beforehand to test the method efficiency. It was seen that improved resolution can be achieved by using a longer SP column in different chemistry. Therefore, Aeris 3.6 um, Widepore XB C8 250×4.6 mm LC column (Phenomenex®, Torrance, California, U.S.A.) was employed in both validation and sample analysis. The FAM method development workflow is illustrated in Fig. 2.

3. Results & discussion

In this study, initially, honey proteins were isolated, concentrated, and cleaned up by elaborately designed sequential ultrafiltration [3]. Afterward, honey invertase and diastase were purified simultaneously by means of FPLC system and using a developed tandem purification procedure. Enzymes of *Apis mellifera* L. origin are not commercially available. Whereby, purified enzymes were used as reference standards (marker authentic proteins), and dominant honey enzymes (diastase and

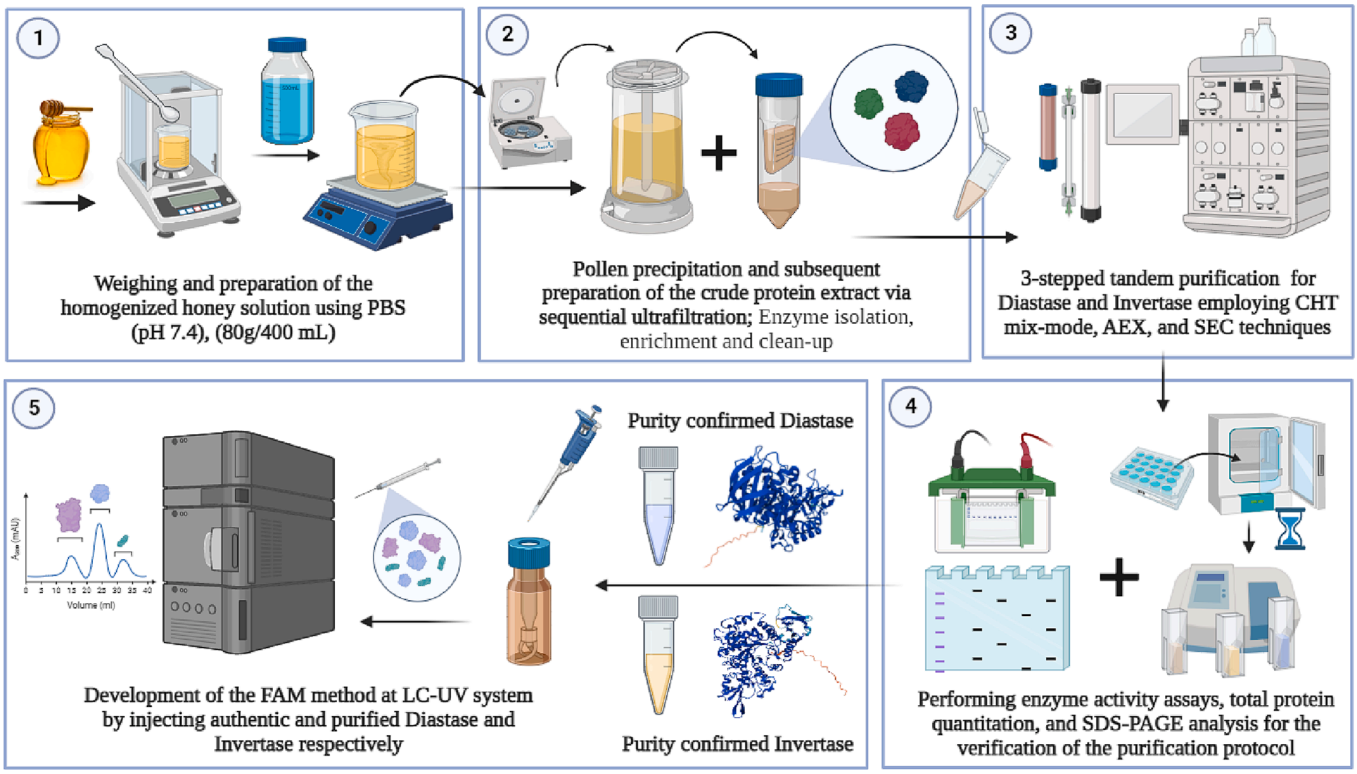

Fig. 2. Summarized description of the FAM method development workflow. 1) Weighing and homogenization, 2) Pollen precipitation followed by protein isolation and enrichment. Further concentration and clean-up performing passive diafiltration. 3) Three steps tandem purification. 4) Conducting enzyme activity assays, protein quantitation, and SDS-PAGE analysis. 5) Development studies of the LC-UV protein profiling method (FAM) by using purified enzymes as authentic markers.

invertase) were labeled in LC-UV chromatogram to discriminate any FD signals.

3.1. Isolation and enrichment of the total honey proteome

The amount of protein in honey is inherently low. Therefore, it is daunting to extract and enrich these proteins and to form a protein-rich crude extract. Before UF as a physical approach, honey proteins were tried to be isolated using chemical techniques. Removal of the chemical agents is mandatory in these methods and denaturant effects of the used agents alter the high-order structure of the enzymes which causes activity loss. Enzyme activity was a pivotal prerequisite for monitoring the purification performance. Unbiased refolding of the enzymes is also unlikely after isolation. Hence, without any further optimization in chemical isolation methods, honey proteins were isolated and concentrated efficiently by serial UF. For this purpose, an authentic pine honey was selected as the method development material owing to its relatively high α-glucosidase (117.1 U/kg) and α-amylase (28.5 Schade U/g) content. Sample was turned into a homogeneous solution using a buffer (10 mM Tris/HCl, pH 7.4, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$). The buffer recipe was constructed to be able to adjust biological pH and required minimum electrolyte concentrations for proteins' stability. MWs of the target enzymes were between 50 and 75 kDa [21,27]. Therefore, UF membrane with 10 kDa MWCO was chosen to get greater recovery and not to extend the UF period. In order to eliminate the plenty of indigenous small molecules, the crude protein solution was subjected to ultra-diafiltration processes using CFU. Total protein assay, diastase activity, and invertase activity assays monitored that a high amount of protein (4.38 mg) and enzyme content (1055 U/kg for invertase and 693 Schade U/g for diastase) was obtained at the end of the isolation. Relevant values for crude extract are given in Table 1.

3.2. Purification workflow for the major honey enzymes

In preliminary experiments, AEX, cation exchange chromatography, glycoprotein affinity chromatography (lentil lectin immobilized to sepharose), hydrophobic interaction chromatography, and CHT multimode chromatography resins have been tested. According to the comparison results, we opted BioRad Foresight™ CHT™ XT, BioRad Enrich™ Q, and BioRad ENrich™ SEC 650 columns. Further, we rigorously investigated purification parameters including the mobile phase compositions and their elution modes. Enzymes need to be purified conserving their properly folded, native structures to get activity results during the purification period. Therefore all process was maintained at 4 °C and denaturant chemicals were not involved in studies. We designed a feasible and sequential purification process by using three steps tandem chromatography. Fig. 1 shows an overall flow diagram of the purification protocol.

In theory, CHT multimode chromatography technique possesses more than one interaction mechanism [22]. While the calcium ions in the resin is a good ligand for metal affinity, the hydroxyl groups in the hydroxyapatite structure can provide a hydrogen bond interaction. Apart from this phenomenon, the other basic interaction type is the cationic protein binding affinity, which is formed by the negatively charged phosphate groups. Most α-amylases are known to be stabilized by Ca^{2+} ions. This mineral is strongly attached to the molecule and it has been thought to be vital for the enzyme's tertiary structure [9]. At this point, diastase, which has calcium binding affinity, was foreseen to be retained by Foresight CHT XT resin with metal affinity interaction. Previously concentrated crude extract was injected into conditioned CHT column and flow-through fractions (invertase) accompanying eluted fractions (diastase) were collected after enzyme activity assay confirmations. Invertase was not observed to be absorbed in CHT and diastase eluted as a single peak at roughly 65 mM [PO_4^{3-}] concentration. As can be seen in Fig. 3(A), CHT chromatography yielded two well-separated responses, designated as diastase (A14-15–16) and invertase (A3-4). While it was seen that calcium ligands in the CHT structure evidently enabled satisfactory bind & elute mode purification for α-amylase, simultaneously, flow-through mode purification was achieved for unbound invertase. It was thought that invertase was not retained either, mainly due to its anionic property and/or lack of metal binding affinity. Subsequently, the activity confirmed fractions corresponding to diastase and invertase were detected in agreement with literature as 55 kDa and 70 kDa protein bands respectively by SDS-PAGE analysis (Fig. 3(F); lane 2–3). In early stages, it has been deduced that invertase has roughly 70 kDa MW and diastase has 55 kDa [20,27] MW under their natural states. Metal affinity chromatography using CHT as an initial purification method has enabled us to obtain higher specific

Table 1
Purification performance results.

Purification performance chart for *Apis mellifera* L. Diastase							
Purification Step	Volume (mL)	Total Protein Conc. (mg/mL)	Total Protein Amount (mg)	Total Diastase Activity (Schade Unit/g = DN[a])	Recovery/ yield (%)	Purification Factor/Fold	Specific Activity (Schade U/mg protein)
Crude extraxt	3.5	1.28±0.04[e]	4.39	693.3±9.2	100.0	1.0	158.7
CHT[b] fraction (A14-A15-A16)	3.0	0.09±0.02	0.22	246.1±5.1	35.5	6.1	960.4
AEX[c] flow-through fraction (A2-A3)	2.0	0.06±0.02	0.17	183.5±3.4	26.5	9.1	1448.0
SEC[d] fraction (A21-A22)	1.0	0.01±0.01	0.01	69.9±1.2	10.1	39.2	6209.1
Purification performance chart for *Apis mellifera* L. Invertase							
Purification Step	Volume (mL)	Total Protein Conc. (mg/mL)	Total Protein Amount (mg)	Total Invertase Activity (U/Kg)	Recovery/ yield (%)	Purification Factor/Fold	Specific Activity (U/mg protein)
Crude extraxt	3.5	1.28±0.05	4.39	1055.0±21.3	100.0	1.0	241.1
CHT flow-through fraction (A3-A4)	2.0	0.20±0.04	0.51	462.9±11.2	43.9	4.2	1015.0
AEX fraction (A14-A15)	2.0	0.11±0.02	0.22	290.8±8.1	27.6	7.6	1836.0
SEC fraction (A18-A19)	1.0	0.02±0.01	0.02	102.1±5.1	9.7	19.9	4788.2

[a] DN; Diastase Number, CHT.
[b] Ceramic Hydroxyapatite.
[c] AEX; Anion Exhange.
[d] SEC; Size Exclusion Chromatography.
[e] Results are expressed as means ± the standard deviation and each measurement was read as triplicate.

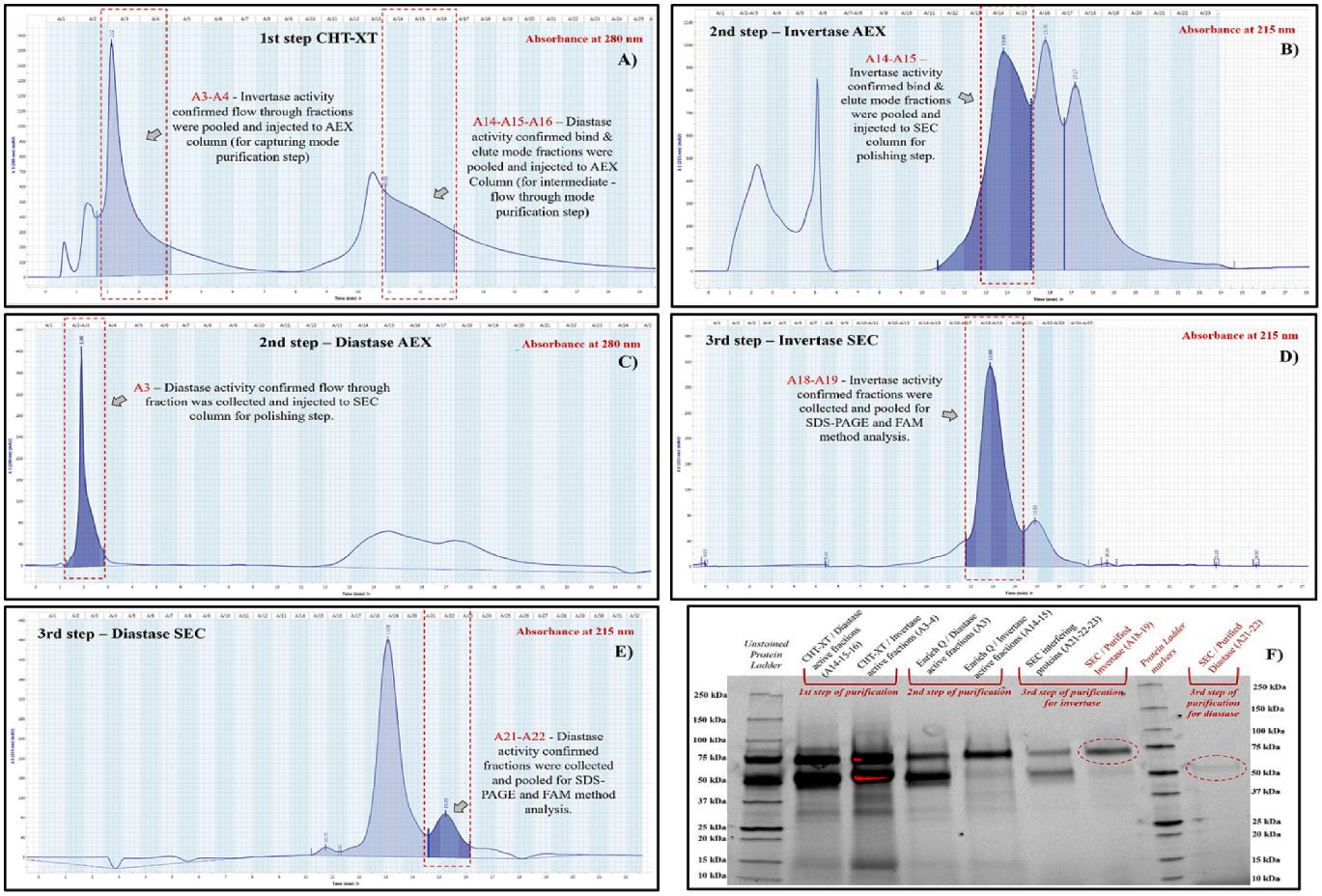

Fig. 3. Purification results. A) CHT chromatography flow-through fraction (A3-4) as unbound molecules including invertase, and eluted fraction (A23-24) as bound proteins including diastase. B) AEX chromatography flow-through fraction represents unbound proteins including diastase (A2-3). C) AEX chromatography eluted fraction represents bound proteins including invertase (A14-15). D) SEC fractionation for diastase (A21-22). E) SEC fractionation for invertase (A18-19). F) SDS-PAGE purity check analysis results for each purification step.

activity, and purer enzyme isolates. Within this strategy, a simultaneous, rapid and effective first step of purification was able to achieve.

In order to eliminate impurities, secondary purification (intermediate purifying step for diastase and capturing step for invertase) was conducted by applying NaCl gradient elution from 0 to 0.5 M on low-salt buffer equilibrated Enrich™ Q anion exchanger. The column named ENrich Q has the strong anion exchanger and quaternary ammonium functional group attached to the polymeric backbone. It was aimed to provide higher resolution due to the 10 μm particle diameter. Diastase activity was detected in the flow-through fraction Fig. 3(C); A2-3 and the gradient elution profile exhibited three discrete peaks for invertase. Invertase activity was recorded at roughly 65 mM NaCl concentration Fig. 3(B); A14-15 after applying a salt gradient. Invertase is a weakly acidic glycoprotein with pI of 5.06 and it undergoes N-linked glycosylation as a post translational modification [31]. The slightly acidic nature of invertase enabled the bind & elute mode AEX purification and removal of impurities, while diastase with its relatively high pI (6.94) and hydrophobicity completed the secondary step purification process in flow-through mode without retention. The SDS-PAGE analysis of the A14-15 fraction revealed an apparent protein band at 70 kDa recognized as invertase (Fig. 3(F); lane 5). The SDS-PAGE image of the flow-through fraction (A2-3) indicated 55 kDa and 75 kDa protein bands (Fig. 3(F); lane 4), and this was criticized as the presence of the interfering proteins such as MRJPs co-eluted with partially purified diastase.

The third and final step as polishing part of the purification workflow was made via gel filtration method. Isocratic elution profile exhibited two representative peaks for both invertase and diastase separately. Purified molecules (Fig. 3(D); A18-19 for invertase and Fig. 3(E); A21-22 for diastase) were further transmitted to SDS-PAGE for final purity check. The MW of invertase in this research was determined as approximately 70 kDa by SDS-PAGE (Fig. 3(F); lane 7) which aligned with the literature. The purified diastase appeared as a single protein band with a MW of approximately 55 kDa (Fig. 3(F); lane 9), conforming with previously published results [21,27]. Purification results as superimposed FPLC chromatograms and image of the SDS-PAGE analysis are given in Fig. 3.

3.3. Enzyme purification performance

Purification performance was tracked during the purification and according to the results, recoveries, purification folds, and specific activities were affirmative for the usage of purified enzymes as analytical standards. Purification performance results were tabulated and given in Table 1.

Enzymes were found to be purified up to 39.2 and 19.9 times with a recovery rate of 10.1% and 9.7% compared to the crude extract for diastase and invertase, respectively. These results were highlighting that these enzymes were purified to near homogeneity. The specific activities of purified diastase and invertase solutions were higher with the results of 6209 Schade U/mg protein and 4788.2 U/mg protein respectively than those of crude extract. Resemble yields were measured for both enzymes but a lower purification factor and specific activity were reported for invertase. We envisaged that the highly heat-labile and fragile structure of invertase might cause denaturation associated with

degradation and thus activity loss over the course of purification. Diastase denoted a relatively pale band at SDS-PAGE analysis and probably a tiny amount of enzyme, recovery loss, and/or dilution effect led to low yield. But in this case, it was more worth trying to obtain diastase as pure as possible and at a sufficient amount to be able to identify the authentic diastase at the LC-UV protein profile.

3.4. FAM method development considerations

To achieve the best analysis efficacy in terms of sensitivity, resolution and reproducibility, the sweet-spots of the sample preparation and LC conditions were all determined. The dilute & shoot approach was used as miniaturized sample preparation methodology because of its simplicity and practicality. LC method development studies were carried out traditionally with water-ACN gradient in RP conditions and by adding different organic modifiers such as TFA, and formic acid. It was figured out that at 0.1% (v/v) concentration of TFA as an ion-pairing agent improved the protein resolution. Owing to the different hydrophobic interaction affinities of the enzymes, RP was preferred as the chromatographic mode of separation. Initially, a polyphenol ligand RP mAb column containing silica particles with an average pore diameter of 450 Å and a particle diameter of 2.7 μm was used as the bioanalytical column. The analytical column with 2.7 μm SP particle produced high resolution and low back-pressure. Among the tested flow rates (0.5, 0.7, and 1.0 mL/min), the 0.7 mL/min flow rate did not cause higher back-pressure and offered sufficient sensitivity under reasonable run time. The mAb column was used initially due to its wider pore size. The large pore diameter of the column enabled the plethora of non-UV absorbing small molecules to be washed out from the column at the early period of the analysis which resulted in eluted protein transitions without any small molecule interference. This allowed us to observe honey proteome surpassingly clear and in full coverage. In this respect, it can be said that the protein profile we displayed is a region where species (*Apis mellifera* L.) and botanical origin specific. The signal at any different retention time in this region is caused by a difference in the honey proteome. While the proteome of authentic honeys from different botanical origins was similar in terms of proteforms, merely the individual areas of the proteins varied. In this regard, the signal at a different retention time enlightens the presence of a foreign protein mainly FD. To promote chromatographic resolution, an alternative column was also tested. For this, Aeris 3.6 um, Widepore XB C8 250×4.6 mm LC column from Phenomenex was evaluated and enhanced resolution has seemed. C8 ligands anchored to 3.6 um SP silica particles and 250 mm of column length produced better analysis efficiency under mild system pressure. For the optimization of sensitivity, varying honey/PBS ratios and injection amounts were compared for dilute & shoot sample preparation. Since no clean-up is applied to the sample within this technique, a higher amount of honey to PBS ratio was posing risk in terms of column overload, clogging, and carryover. In addition, the areas for each protein peak did not increase linearly with ascending honey/PBS ratios. Thus, 2 g of honey homogenized with 10 mL of PBS and 20 μL of injection volume was decided.

The developed method was verified using adulterated and authentic samples. A wide variety of expression organisms such as fungi, actinomycetes, animals, and plants can produce α-amylase. The most used and relatively cheap FD alternatives in the market originated from *Bacillus lichenifromis*, *Aspergillus oryzae*, and barley. The developed method has succeeded in distinguishing FDs from the native honey proteome, even though they come from different origins. The UV signals of the purified diastase and invertase enzymes were averagely recorded at 13.9 and 14.9 min respectively (Fig. 4(E)). On the other hand, FDs in manipulated samples responded at 14.4 min which made it possible to distinguish fraudulent honey seamlessly. The obtained chromatograms are given in Fig. 4.

Other than FD entity, it was observed that major honey proteins diluted because of the syrup addition Fig. 4(C and E). This unprecedented interpretation capability also gave us an opportunity to differentiate between counterfeit and adulterated honey. While authentic honey enzymes can be degraded by misapplications such as elongated shelf life and abnormal heat treatment, characteristic honey proteins like MRJPs are relatively more durable. In the case of syrup addition, enzymes will be diluted concurrently with other honey proteins. This reflects the syrup addition, otherwise, misapplications lead solely the enzyme loss and the other honey proteins remain consistent. This paved the way to make a data-driven comment on the use of FD to mask the values of non-fresh honeys or to compensate for the dilution effect from adulteration.

3.5. Method performance results

The FAM method was validated to demonstrate its applicability for the qualitative identification of the FDs. An in-house validation procedure has been structured and carried out including linearity, precision as intra-day repeatability, and accuracy/selectivity as identification capability of the FD. For the specificity and selectivity tests, authentic honeys from various botanical origins were tested to investigate if any protein peaks are present that may interfere with the authentic proteins. Due to the absence of the round-robin test for accuracy, we spiked the known amounts of FDs from different expression microorganisms into the authentic honeys and assessed the obtained chromatograms. Any apparent signal at the expected retention time of the FD was interpreted as the method being accurate to identify adulteration. For this, stock solutions of each FD (*Aspergillus oryzae, Bacillus licheniformis*, and barley) was spiked individually into the authentic honeys from different botanical origins at linearly increased concentrations.

The intra-day precision and accuracy were determined from three independent preparations. The linearity curves were formed with quadratic fit and $1/x^2$ weight for each FD variant. The findings demonstrated excellent assay performance and according to the validation results of the FAM method, precise (harmonized intra-day RSD_r (CV%); 1.27%), and linear (harmonized R^2 >0.9950) results were able to obtain for each FD variant and honey from different botanical origins. Spiking linear concentrations of FD highlighted that higher amounts of the FD enzyme can be identified in a linear fashion accurately. Specificity test results pointed out similar protein peak distributions only with varying signal abundancies. Method performance results are shown in Table S2.

3.6. Sample analysis results

We investigated a total of 202 samples over 3 consecutive years using both the developed FAM method and diastase activity assays to see a potential correlation. Unequivocally, the apparent FD peaks were identified almost in all diastase adulterated honeys. It was figured out that the presence of FD was correlated in samples with atypically high diastase values (DN >30). 74 out of 202 samples were diagnosed to be adulterated using direct FD or sugar syrups that contain FD and these findings revealed the growing problem of honey authenticity. Trend analysis has been accomplished to see the increasing adulteration frequency. As given in Table 2, in 2020, FD was solely reported in 1 sample among the analyzed 30 samples. Astoundingly, it was observed that, with the beginning of 2021 year, FD containing honey samples increased. In 2021, 48 out of 124 honeys were reported as FD adulterated, and in the year 2022, more dramatically, 25 samples were found to be FD-containing and this reflected the 52% positive ratio for the sample set of 2022. Assuming the process diversities of the used sugar syrups are not changed, this was assessed as honeys are being adulterated by the addition of FD rather than the FD-containing syrup addition. The follow-up study also proved that to hide the fraud successfully, in recent years, diastase activity is trying to be managed not to present abnormally high levels of DN. It has been experienced that, favorable diastase activities were found with corresponding FAM positivity and the percentage of

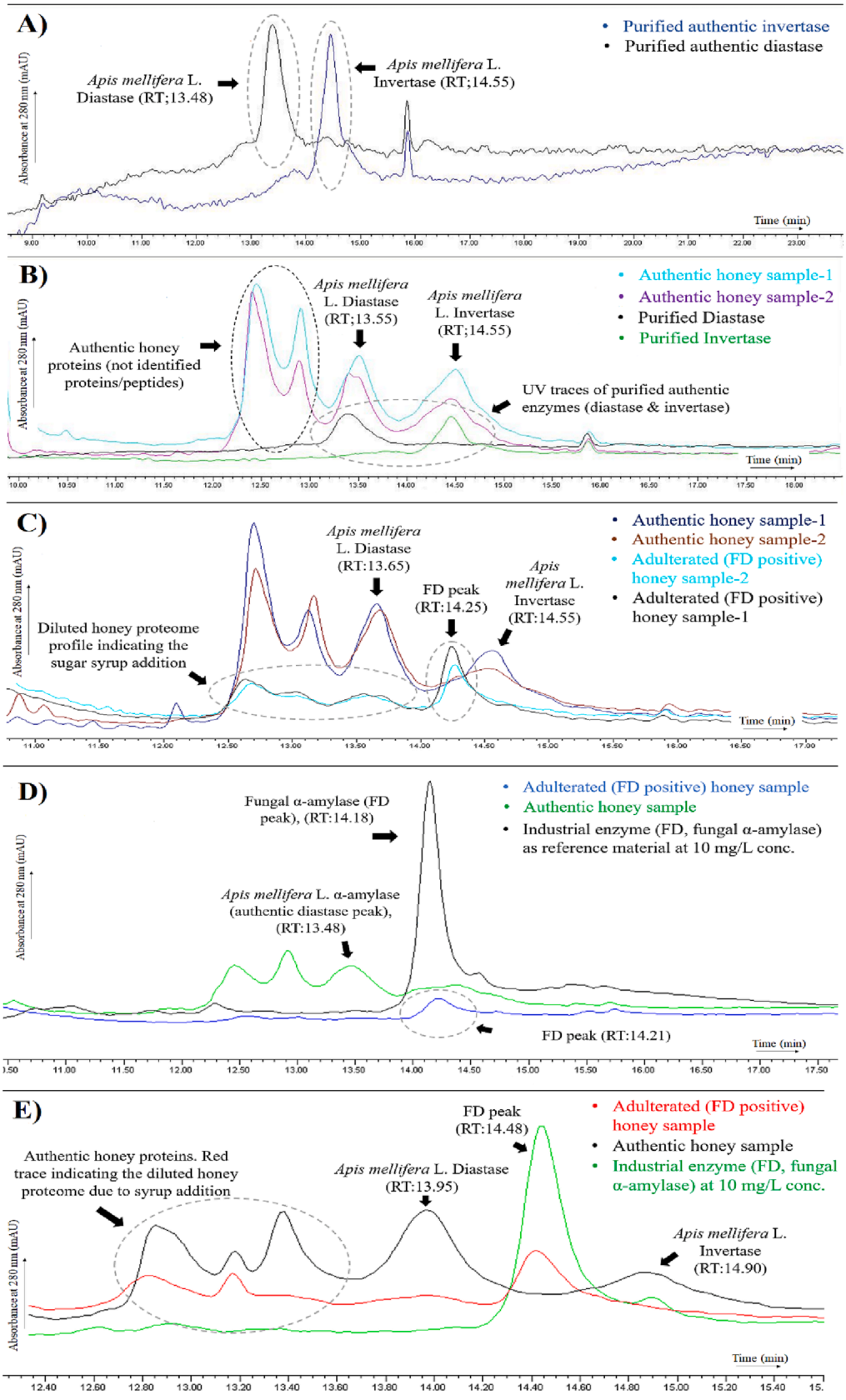

Fig. 4. LC-UV chromatograms of the FAM method. A) LC-UV traces of purified invertase and diastase. B) Overlaid LC-UV traces of the comparison study regarding authentic samples and purified honey diastase and invertase. C) Overlaid LC-UV traces of the comparison of authentic samples with adulterated honeys containing FD. D) Overlaid LC-UV traces of an authentic sample, industrial α-amylase obtained from the fungal origin (*Aspergillus oryzae*), and adulterated sample as a comparison. E) Overlaid LC-UV chromatograms of the adulterated honey, an authentic sample, and industrial α-amylase after the method application using Phenomonex Aeris protein column.

Table 2
Samples failing the FAM test by year and their corresponding diastase activities.

Sampling Year	Sample Code	Adulteration assesment according to FAM[a] analysis; Pass/Fail	Diastase Acitvity (Schade U/g, DN[b])	Sampling Year	Sample Code	Adulteration assesment according to FAM analysis; Pass/Fail	Diastase Acitvity (Schade U/g, DN)
2020	FAM-2020–21	Fail	14.3±0.2[d]	2021 (cont'd)	FAM-2021–87	Fail	50.0±0.7
2021	FAM-2021–10	Fail	20.0±0.2		FAM-2021–89	Fail	50.0±0.8
	FAM-2021–11	Fail	16.7±0.5		FAM-2021–92	Fail	50.0±0.3
	FAM-2021–12	Fail	14.3±0.6		FAM-2021–98	Fail	70.0±0.8
	FAM-2021–16	Fail	17.9±0.5		FAM-2021–101	Fail	33.3±0.5
	FAM-2021–24	Fail	50.0±0.2		FAM-2021–104	Fail	50.0±0.7
	FAM-2021–26	Fail	AUL[c]		FAM-2021–105	Fail	33.3±0.3
	FAM-2021–28	Fail	50.0±0.5		FAM-2021–106	Fail	AUL
	FAM-2021–32	Fail	45.5±0.1		FAM-2021–111	Fail	AUL
	FAM-2021–35	Fail	43.5±0.4		FAM-2021–116	Fail	AUL
	FAM-2021–36	Fail	AUL		FAM-2021–121	Fail	31.3±0.5
	FAM-2021–37	Fail	43.5±0.5		FAM-2021–122	Fail	33.3±0.5
	FAM-2021–38	Fail	33.3±0.5	2022	FAM-2022–05	Fail	29.4±0.5
	FAM-2021–40	Fail	70.0±0.8		FAM-2022–06	Fail	18.5±0.3
	FAM-2021–41	Fail	50.0±0.2		FAM-2022–08	Fail	21.7±0.5
	FAM-2021–42	Fail	33.3±0.4		FAM-2022–09	Fail	31.3±0.4
	FAM-2021–44	Fail	70.0±0.8		FAM-2022–11	Fail	13.2±0.5
	FAM-2021–46	Fail	70.0±1.2		FAM-2022–14	Fail	9.1±0.2
	FAM-2021–47	Fail	AUL		FAM-2022–15	Fail	4.7±0.1
	FAM-2021–48	Fail	35.7±0.7		FAM-2022–16	Fail	33.3±0.7
	FAM-2021–55	Fail	70.0±0.7		FAM-2022–17	Fail	50.0±0.6
	FAM-2021–57	Fail	70.0±1.9		FAM-2022–18	Fail	64.0±0.2
	FAM-2021–62	Fail	70.0±0.9		FAM-2022–20	Fail	38.5±0.3
	FAM-2021–63	Fail	33.3±0.3		FAM-2022–22	Fail	38.5±0.6
	FAM-2021–65	Fail	70.0±0.2		FAM-2022–24	Fail	64.0
	FAM-2021–67	Fail	AUL		FAM-2022–25	Fail	13.9±0.3
	FAM-2021–69	Fail	70.0±0.5		FAM-2022–27	Fail	64.0±0.9
	FAM-2021–70	Fail	50.0±0.5		FAM-2022–28	Fail	38.5±0.3
	FAM-2021–71	Fail	70.0±0.4		FAM-2022–31	Fail	64.0±0.9
	FAM-2021–72	Fail	35.7±0.2		FAM-2022–32	Fail	64.0±1.1
	FAM-2021–74	Fail	45.5±0.2		FAM-2022–33	Fail	64.0±0.8
	FAM-2021–75	Fail	31.3±0.5		FAM-2022–38	Fail	64.0±1.7
	FAM-2021–79	Fail	45.5±0.5		FAM-2022–39	Fail	50.0±0.7
	FAM-2021–82	Fail	50.0±0.3		FAM-2022–40	Fail	21.7±0.4
	FAM-2021–83	Fail	47.6±0.2		FAM-2022–43	Fail	50.0±0.7
	FAM-2021–84	Fail	50.0±0.5		FAM-2022–44	Fail	21.7±0.4
	FAM-2021–85	Fail	50.0±0.5		FAM-2022–48	Fail	50.0±0.4

[a] FAM method; Foreign Amylase Monitoring.
[b] DN; Diastase number.
[c] AUL; Above the upper detection limit of phadebas method.
[d] Diastase activity results are expressed as means ± the standard deviation and each honey sample was run in triplicate.

this matching was higher with increasing years. Summarized results are given in Table 2 and detailed results are given in Table S3 (S.I.).

As far reaching implications, it was proven that the presence of FD may not necessarily accompany a high diastase value. As encountered in the FAM-2022–15 sample with a 4.7 DN, the activity of exogenously added or syrup derived FD may be low. Remarkably, FD activity may be lessened due to the extended shelf life or heat treatment, and therefore it may not increase the total diastase activity much. This drew attention to the necessity of probing the presence of FD even in honeys with conceivable or low diastase activity. It ought to be stated that FD may also be detected as syrup residue. In the production of syrups, enzymes can be used as immobilized format in the process for enzymatic starch hydrolysis (amylases) or sucrose inversion (glucosidases). Alternatively, FD or BFF components that may be present in the syrup can be diminished by using IEX resins. Another enzyme removal strategy is to inactivate (denature) enzymes by applying high temperature. However, these applications will not increase the diastase activity due to the removal or inactivation of FD but may leave residues. Furthermore, if amylases except heat-stable FD are added to honey, and the product is subjected to a long shelf life, the total diastase activity may decrease, making it difficult to determine the FD additive over the total activity. FD can be added to honey in excess, but the amylase activity of the added product may be negligible. Similarly, by adding a very small amount of FD with high amylase activity, aberrant elevation of total diastase value can be modulated and adulteration can be hidden. All of these underscore that FD determination by activity assays can cause false negatives to be reported. Observing the specific response of FD with alternative techniques is the most dependable way. In other words, there may not be adulteration merely in the form of FD spiking. Making this differentiation is not easy with current methodologies. Nevertheless, since Codex Alimentarius indicates the primary quality criterion statement as 'Honey sold as such shall not have added to it any food ingredient, nor shall any other additions be made other than honey' [12], the presence of all kinds of FD in honey outlines the detection of unnatural honey. There is not any set upper limit for diastase activity as neither quality nor analytical criteria. Thereby, FD-added honey cannot be claimed as adulterated even if exceeded activity results are seen. In our opinion, setting only a lower limit by Codex (DN should be higher than 8 Schade U/g) is not totally reflecting the quality and it is not fully convenient in terms of identification of the enzyme adulteration.

4. Conclusion

In this study, as an up-to-date contribution to the literature, an alternative purification protocol rather than widely preferred techniques in protein purification for honey diastase and invertase was presented. CHT resin usage as the initial/capturing step of the purposed enzymes in honey is new to the literature and this enabled to get enzymes at two separate fractions simultaneously at the end of the single injection. The uncomplicated dilute and shoot sample preparation and LC method offering enhanced chromatographic resolution were optimized for FD detection in the FAM method. The developed FAM method was able to detect the presence of FD in adulterated samples accurately. The analytically validated method is cost-effective, rapid, accurate, and effortless to use compared to enzymatic activity or proteomic based approaches. This presented methodology will reduce false negative test results and also improve the overall reliability of the colorimetric diastase assays. The novel method and performed trend analysis also uncovered the prevalence of FD in honey and presented an actionable dataset as valuable information on mimicked honey being significant for food safety. It was revealed that honey adulterants targeting increased diastase value are getting a major problem for secure honey commerce.

CRediT authorship contribution statement

İsmail Emir Akyıldız: Writing – original draft, Methodology, Investigation, Conceptualization. **Özge Erdem:** Methodology, Validation, Formal analysis. **Sinem Raday:** Writing – review & editing. **Sezer Acar:** Visualization, Resources, Investigation. **Dilek Uzunöner:** Data curation, Formal analysis. **Emel Damarlı:** Project administration. **Ece Kök Yetimoğlu:** Supervision.

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.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109039.

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