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Detection of Honey Adulteration by Liquid Chromatography—High-Resolution Mass Spectrometry: Results from an EU Coordinated Action

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Abstract: Honey, a sweet and nutritious food produced by honeybees, is extensively consumed by humans due to its potential health benefits. Unfortunately, the adulteration of honey with inexpensive sugar syrups is a prevalent issue. Verifying the authenticity of honey is crucial for maintaining its quality and safety standards. The aim of this study was to identify the illicit addition of sugar syrups to honey imported into the European Union (EU). The European Commission's Joint Research Centre (JRC) has employed different analytical approaches to detect several markers of adulteration in honey; however, this paper mainly focuses on the use of Liquid Chromatography–High-Resolution Mass Spectrometry (LC-HRMS). Two qualitative methods were developed to detect mannose (Man), difructose anhydride III (DFA III), 2-acetylfuran-3-glucopyranoside (AFGP), and oligo-/polysaccharides with degrees of polymerization (DPs) of 6 to 11. Out of the 320 honey samples provided by the authorities of the participating EU Member States, 147 (46%) were suspicious for non-compliance with the EU Honey Directive 2001/110/EC, mostly due to the presence of mannose and oligo-/polysaccharides. As a result, the development and standardization of sophisticated and universally recognized testing procedures will increase the capability of official control laboratories to detect honey adulteration and will serve as a powerful preventive measure against fraudulent practices in the global honey market.

Keywords: honey; adulteration; LC-HRMS; consumer protection; food fraud

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1. Introduction

Honey, a sweet and highly nutritious substance produced by the foraging activity of honeybees, is widely consumed by humans as it is claimed to possess several therapeutic effects due to its antioxidant, antimicrobial, and anti-inflammatory properties [1–3]. Honey is a very complex matrix, and it includes a wide range of nutrients such as carbohydrates, proteins, enzymes, organic acids, phenolic substances, minerals, and amino acids. Of those nutrients, carbohydrates, mainly fructose (Fru) and glucose (Glc), make up around 80% [4,5]. The relatively high price, limited production, and complex composition of honey have provided significant incentives for honey adulteration, affecting both producers and consumers. The most common honey adulteration involves the addition of inexpensive sugar products, as their carbohydrate profiles can be easily manipulated to resemble the

carbohydrate profile of honey [6,7]. Sugar syrups from corn [8,9], rice [10], sugar cane, and sugar beet [11–13] produced by applying acids and enzymes [14–16] such as high fructose corn syrups (HFCS) have been detected as adulterants in honey [9,14,17]. Honey can also be indirectly adulterated through the incorrect feeding of honeybees with complementary feeds during the main nectar flow period [18,19]. Recently, there has been increasing interest in the control of honey quality and in the development of new methods to detect honey frauds. Yet, identifying honey adulteration can be challenging because the natural composition of honey varies greatly based on its botanical and geographical origin, and because the syrups used to adulterate honey have a chemical composition similar to that of natural honey [6,7]. The European Union (EU) Honey Directive 2001/110/EC [20] seeks to maintain the integrity of honey as an unprocessed raw agricultural product, prohibiting alterations to its chemical composition. Within the EU, market demand for honey exceeds domestic production, leading to significant imports. [21]. Unfortunately, not all honey placed on the market is authentic. National control authorities, producer organizations, as well as consumer protection associations have consistently expressed concern about the availability of honey that does not adhere to regulatory standards in the market [21,22].

The determination of honey authenticity is of importance to ensure its quality and safety. Therefore, a coordinated action at EU level was organized to collect data on the prevalence of non-compliant honey entering the EU through sampling and analysis, which was aimed at directing further investigations within the EU. The action was initiated and coordinated by the Directorate-General for Health and Food Safety (DG SANTE) of the European Commission and implemented by the members of the EU Food Fraud Network (EU FFN), who received the technical assistance of the European Commission's Joint Research Centre (JRC) and the support of the European Anti-Fraud Office (OLAF) [21]. The JRC's responsibility was to evaluate the honey samples provided by the competent authorities of the participating EU Member States using various analytical methods. The aim of this paper is to discuss the results obtained from the EU coordinated action [21], with an emphasis on the analytical techniques, in particular Liquid Chromatography–High-Resolution Mass Spectrometry (LC-HRMS), used for the analysis of markers indicative of the presence of exogenous sugar syrups.

2. Materials and Methods

2.1. Reagents and Chemicals

Ultra-pure water was obtained from a Milli-Q water purification system with a 0.22 µm hydrophilic membrane filter from Merck (Millipore, Burlington, MA, USA). Acetonitrile (ACN) was purchased from VWR (Radnor, PA, USA). Cesium acetate (CsOAc), 25% ammonium hydroxide (NH_4OH), mannose, and maltodextrin (dextrose equivalent 13.0–17.0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Difructose anhydride III (DFA III) was obtained from Wako Chemicals Europe GmbH (Neuss, Germany), and 2-acetylfuran-3-glucopyranoside (AFGP) from Toronto Research Chemicals (North York, ON, Canada).

2.2. Honey Sample Collection

A total of 320 consignments of honey imported into the EU were sampled between January 2021 and June 2022 by fifteen EU Member States (Belgium, Bulgaria, Czech Republic, Germany, Denmark, Greece, Spain, France, Hungary, Ireland, Italy, Lithuania, Poland, Romania, and Sweden), along with Switzerland and Norway (Figure 1).

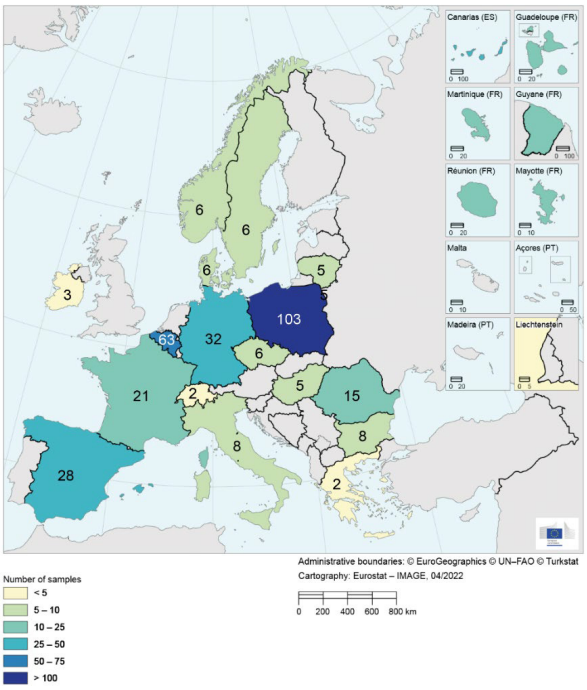


Figure 1. EU–Member States participating to the EU–coordinated action; figure taken from [21].

The information provided by the competent authorities indicated that the vast majority of the honey samples (84%) were collected at Border Control Points (BCPs), while the remaining samples were obtained either from the facilities of honey packers and distributors or from unspecified locations. Honey consignments came mainly from China (89), followed by Ukraine (74), Argentina (34), Mexico (22), Brazil (18), and Turkey (15), as illustrated in Figure 2, and most of them were labelled as either polyfloral honey (77%) or monofloral honey (11%). The botanical origin of the remaining honey consignments was not specified [21].

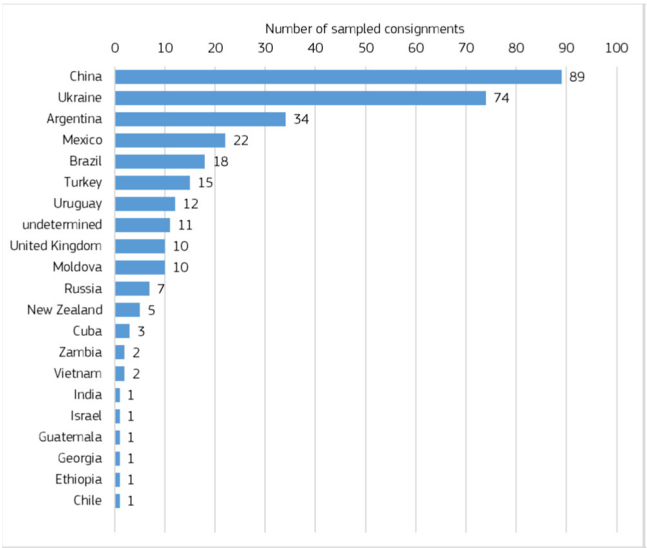


Figure 2. Geographical origin of sampled honey consignments; figure taken from [21].

2.3. Sample Preparation for LC-HRMS Analysis

Samples (ca. 1 g) were weighed into 50 mL screw-cap centrifuge tubes, and 10 mL of ACN:Water (50:50, *v*/*v*) was added. The mixture was vortex-mixed for 2 min and then further agitated on a shaker for another 5–10 min. Afterwards, the solutions were filtered

through 0.2 µm filters into new tubes. To prepare the injection solutions for LC-HRMS, 50 µL of each sample was added to 450 µL of ACN:Water (50:50, *v*/*v*) and filtered again with Mini-UniPrep filter vials (Whatman, cytiva.com) with 0.2 µm nylon membranes. These vials were then placed into the auto liquid sampler.

2.4. LC-HRMS Analysis

Two qualitative analytical methods were developed to detect different markers of adulteration in honey. For both methods, the analyses were performed on an LC consisting of two LC-20AD XR pumps, a CTO-30A oven, and a SIL-30AC ALS (all Shimadzu, Duisburg, Germany) coupled to a high-resolution MS (Orbitrap Elite—ThermoFisher Scientific, Waltham, MA, USA) with the heated electrospray ionization (HESI) source. Acquisitions were in full scan positive mode with a scan range from *m*/*z* 150 to 2000 and resolving power of 60,000 at *m*/*z* 400. Chromatographic separation was afforded by an Aquity UPLC BEH Amide 1.7 µm, 2.1 × 100 mm column (Waters, Milford, MA, USA). A column temperature of 80 °C was maintained throughout the run. Mobile phases consisted of 0.02% of NH_4OH (25%) and 50 µM CsOAc in water (Solvent A) and 0.02% NH_4OH (25%) and 50 µM CsOAc in ACN (Solvent B). The ALS flow paths were cleaned in between runs with 0.02% NH_4OH (25%)/50 µM CsOAc in ACN:water (50:50, *v*/*v*).

In positive ESI mode, saccharides are only accessible as metal adducts. Cesium (Cs) was chosen as a metal ion (A) because it is monoisotopic, which means only a single ion species is formed, and (B) because of its high molecular weight of 132.90 amu; it shifts the *m*/*z* of its adducts to mass ranges with less background from, e.g., solvent clusters. Sodium (Na, 22.99 amu), a ubiquitous metal ion found in all mobile phases stored in glass containers, is also monoisotopic, but would not shift the saccharide signals to much higher mass ranges because of its much lower molecular weight. Adding CsOAc to the mobile phase effectively suppresses sodiation and shifts the hexose signals to *m*/*z* 312.97 [23]. The basic pH of the mobile phase was chosen to prevent slow mutarotation of the analytes from possibly causing peak broadening or even peak splitting.

Method 1 was used for the analysis of mannose, AFGP, and DFA III. The volume injected was 1 µL, and a multi-step gradient at a flow rate of 0.2 mL/min was used as follows for Solvent B: 0 min, 85%; 5 min, 80%; 5.01 min, 40%; 8 min, 40%; 8.01 min, 85%; 10 min, 85%. Method 2 was used for the analysis of oligo-/polysaccharides with a degree of polymerization (DP) of 6 to 11. The volume injected was 20 µL, and a multi-step gradient at a flow rate of 0.2 mL/min was used as follows for Solvent B: 0 min, 50%; 5 min, 20%; 7 min, 20%; 7.01 min, 50%; 9 min, 50%. Table 1 shows the accurate masses of the cesium adducts for mannose, AFGP, DFA III, and DP 6 to 11.

Table 1. Accurate masses of mannose, DFA III, AFGP, and oligo-/polysaccharides cesium adducts.

Compound	ESI^+
	$[M + Cs]^+$ *m*/*z*
Mannose	312.9682
DFA III	457.0105
AFGP	420.9894
DP 6	1123.2323
DP 7	1285.2852
DP 8	1447.3380
DP9	1609.3908
DP10	1771.4436
DP11	1933.4965

Compounds were identified by comparison to quality control (QC) samples composed of marker-free honey into which mannose, AFGP, DFA III, and oligo-/polysaccharides were added. For the oligo-/polysaccharides, maltodextrin was added to provide an indication of the retention time of DP 6 to 11 without knowledge of the individual amounts. The QC samples were measured at the beginning and the end of the sequence and after every tenth injection.

2.5. EA/LC-IRMS and ^{1}H-NMR Analyses

Elemental analysis (EA)–Isotope Ratio Mass Spectrometry (IRMS) of proteins isolated from honey was executed for all investigated samples to obtain a sample-specific baseline of $^{13}C/^{12}C$ ratios ($\delta^{13}C$ value). With LC-IRMS, the $\delta^{13}C$ values of individual saccharides were obtained [24,25]. The determined $\delta^{13}C$ values were then used according to the paper of Elflein and Raezke [25]. The EA-IRMS analyses were executed with an EA IsoLink CNSOH interfaced through a Conflo IV to a Delta V Advantage IRMS. The LC-IRMS consisted of a Dionex UltiMate 3000 coupled to another Delta V Advantage IRMS through the LC Isolink interface (all ThermoFisher Scientific, Waltham, MA, USA).

As a confirmatory analysis, Proton-Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy was applied in case mannose was identified in honey samples by LC-HRMS. The measurements were conducted with a 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). On an individual basis, NMR-based profiling in combination with a commercially available database and software tool (Bruker Honey Profiling™ [26]) was used as supportive evidence prior to making a final decision on the presence of mannose in honey.

3. Results and Discussion

Honey available for consumers in the EU internal market must comply with Council Directive 2001/110/EC, which includes specific requirements about the composition of honey, as listed in its Annex II [20]. Consequently, the detection of an adulteration marker in a honey sample suggests that the honey may be suspicious for not being in compliance with the requirement that honey should not contain any added ingredients, such as sweeteners or preservatives, other than honey itself. In particular, in the present study, a sample was considered as being suspicious of adulteration with a sugar syrup if at least one of the compounds analyzed was detected by different techniques. Detection of honey adulteration with addition of sugar syrups was the primary aim of the coordinated action. Potential adulteration related to geographical or botanical origin was not taken into account.

The LC-HRMS technique's advantage lies in its ability to detect various adulteration markers simultaneously [27,28]. Two qualitative LC-HRMS methods were developed to detect mannose, DFA III, AFGP, and oligo-/polysaccharides with DP between 6 and 11 in honey samples. Figure 3 shows LC-HRMS chromatograms of two suspicious honey samples, where AFGP, DFA III, mannose (B), and DP 6 to 11 (D) were detected, and two non-suspicious honey samples without AFGP, DFA III, mannose (A), or DP 6 to 11 (C).

Oligo-/polysaccharides and mannose were the markers which most frequently flagged suspicious honey, either together or as individual markers. In 44% of suspicious cases, as many as two to five marker substances were present.

Mannose is an epimer of glucose, which is a natural monosaccharide that occurs in certain plants (e.g., jujube) or via hydrolysis of plant wall material (hemicellulose) [29]. Mannose in blossom honey is considered as an indicator of the presence of starch-based sugar syrups or honeys treated with ion exchange resins (purifying process) [30]. It is generally considered a potential marker of adulteration, but it is recommended to include additional markers in the conformity assessment in case mannose is detected in

honey [31]. It is reported that certain honeys, e.g., linden, eucalyptus, jujube, or chestnut, could naturally contain mannose [30].

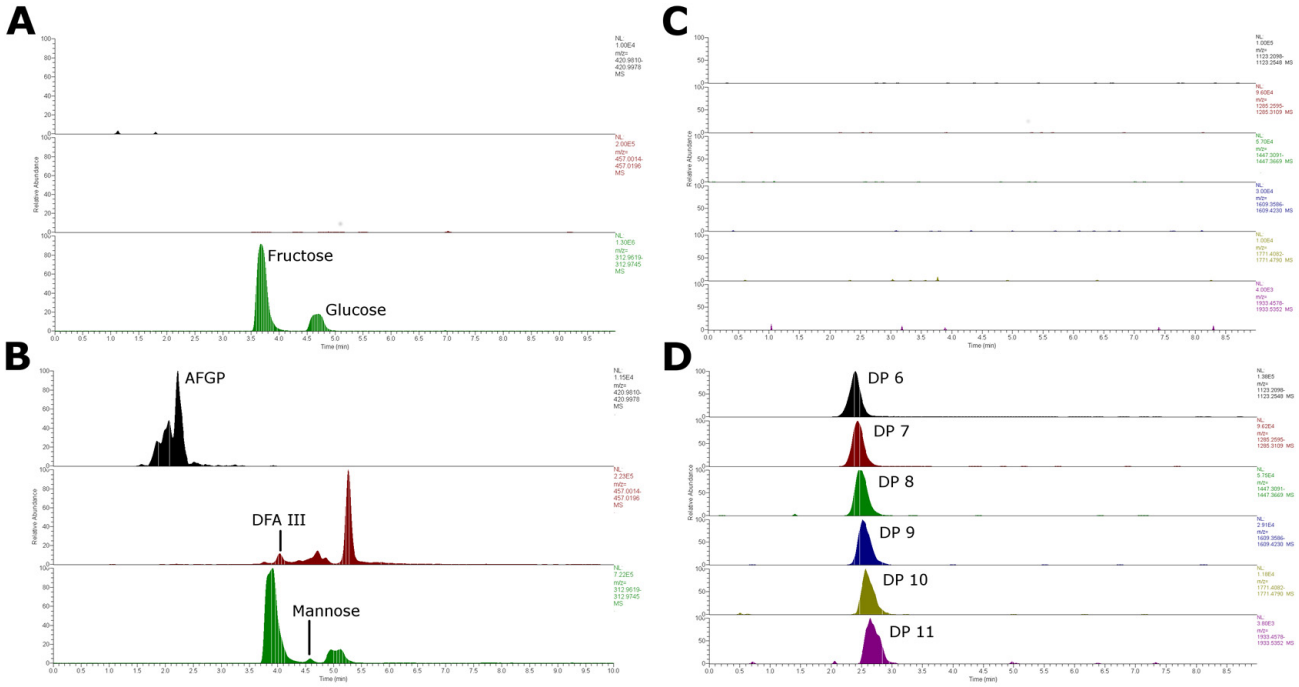


Figure 3. Analysis of honey authenticity: extracted ion current chromatograms showing a non-suspicious honey sample (**A**) and a honey sample suspicious for adulterations (**B**) with detection of AFGP, DFA III, and mannose. Similarly, (**C**) shows a non-suspicious honey sample, and (**D**) shows a suspicious honey sample where DP 6 to 11 was detected, indicating possible adulteration.

Oligo-/polysaccharides with DP 6 to 11 are considered as an indicator of adulteration with starch-based sugar syrups and assumed to be the consequence of partial enzymatic degradation of starch and/or incomplete elimination of them during syrup purification [8,9,32]. However, the origin of oligosaccharides and polysaccharides with a DP higher than 6 or 7 in authentic honey remains uncertain. While it seems to be generally accepted that such high DP saccharides are not found in nectars, the process and extent of their potential formation in honey are not well understood. It is speculated that they may be the result of non-enzymatic transglycosylation reactions (sugar reversion), although only oligosaccharides up to DP 6 were observed in honey model solutions [33]. Additionally, honeydew honeys may contain oligosaccharides, which are produced by aphids. They do not only feed on forest trees, but may also attack grain, e.g., *Melanaphis sorghi* attacking sorghum [34]. Detection markers of honey adulteration also included DFA III and AFGP. DFAs are pseudo-disaccharides produced by condensation of two fructose molecules by means of a caramelization reaction, which takes place during heating of sugars or sugar-rich foodstuffs [14,35]. AFGP is a characteristic compound found in rice syrups and, so far, not in honeys [10]. AFGP is also known as the Special Marker for Rice Syrup (SMR) [36]. The detection of AFGP and DFA III suggested the addition of rice syrups into honey, and of HFCS and inverted syrup (IS), respectively [14,35].

For most samples flagged as suspicious because of mannose detection by LC-HRMS, ^{1}H-NMR analysis verified the presence of the marker. NMR spectroscopy has gained traction in recent years as a potentially promising alternative for the analysis of authenticity and provenance of honey samples; yet, few studies have been published on NMR-based detection of sugar adulterants in honey [37,38]. One advantage of NMR is that it does not require complicated sample preparation or separation of components prior to the actual

analysis, and the determination of very different chemical species can be performed in a single experiment. NMR provides structural information with excellent repeatability and reproducibility. One limitation of NMR is its lower sensitivity in comparison to LC-HRMS [37].

The addition of sugar syrups can also be detected with IRMS. The $\delta^{13}C$ values of all constituents in natural honey should vary only within a narrow range from −23.5‰ to −27.5‰, with an average of −25.4‰. This will be altered if sugar syrups are added. The greatest alterations occur if corn or sugar cane (C4 plants, $\delta^{13}C$ values ranging from −9‰ to −18‰ [24,39]) is the source, and lesser ones occur if sugar beet, rice, or wheat (C3 plants, $\delta^{13}C$ values ranging from −21‰ to −32‰) are the sources of the syrup. Reports in the literature [40] claim that relying solely on $\delta^{13}C$ values of sugars is inadequate for confirming the presence of adulterants from C4 plants in honey. Instead, isolating honey proteins and measuring their $\delta^{13}C$ values by EA-IRMS can enhance the ability to detect adulteration. The addition of syrups would then result in $\delta^{13}C$ values of the honey saccharides, as determined by LC-IRMS, that deviate from the protein value. This approach was developed by Cabañero et al. [41] and ultimately further improved by Elflein and Raezke [25]. They demonstrated that isotopic ratio differences between individual sugars and/or honey protein could be used as authenticity criteria to detect honey adulteration with C4 and even C3 sugars.

In the course of our investigation into honey adulteration, we observed that not all analytical techniques uniformly confirmed the suspicion of adulteration for every sample tested. In some samples flagged as potentially adulterated by LC-HRMS, ^{1}H NMR and EA/LC-IRMS confirmed the finding, but in certain instances, discrepancies arose when samples considered non-suspicious by LC-HRMS were found to be suspicious by EA/LC-IRMS. This variability underscores the importance of employing a multi-analytical approach when examining honey for adulteration. By utilizing several complementary techniques simultaneously, we can achieve a more robust and reliable assessment of the honey's authenticity. The combined use of LC-HRMS, ^{1}H-NMR, and LC-IRMS enhances the detection capabilities for various adulterants, thus providing a comprehensive picture of the honey's purity and ensuring a higher level of confidence in the results.

Results regarding the number of samples considered suspicious of adulteration are shown in Figure 4. A total of 320 samples were analyzed, and 147 (46%) were found to be suspicious for being non-compliant with the provisions of the EU Honey Directive 2001/110/EC [20].

The percentage of suspicious honey samples was substantially higher than the results from a previous EU-coordinated control plan during 2015–2017, which reported non-compliance in only 14% of the samples applying the EA/LC-IRMS [21]. The increased detection of potential adulteration could be attributed to the use of additional chemical markers in the recent evaluation.

In absolute numbers, most suspicious consignments were traced back to China (66 out of 89 samples, 74%). In comparison, samples imported from the United Kingdom (UK), which, at the time of the sampling, was no longer a member of the EU, showed the highest rate of suspicion with 100% (10 out of 10), followed by Turkey with 93% (14 out of 15). Nonetheless, the accessible traceability data imply that the UK samples may represent honey originally produced in other countries and subsequently processed in the UK prior to exportation to the EU.

Of the EU Member States that participated in the coordinated action, Romania had the lowest rate of suspicious samples (3 out of 15, 20%), while the highest rate was observed for France (17 out of 21, 81%).

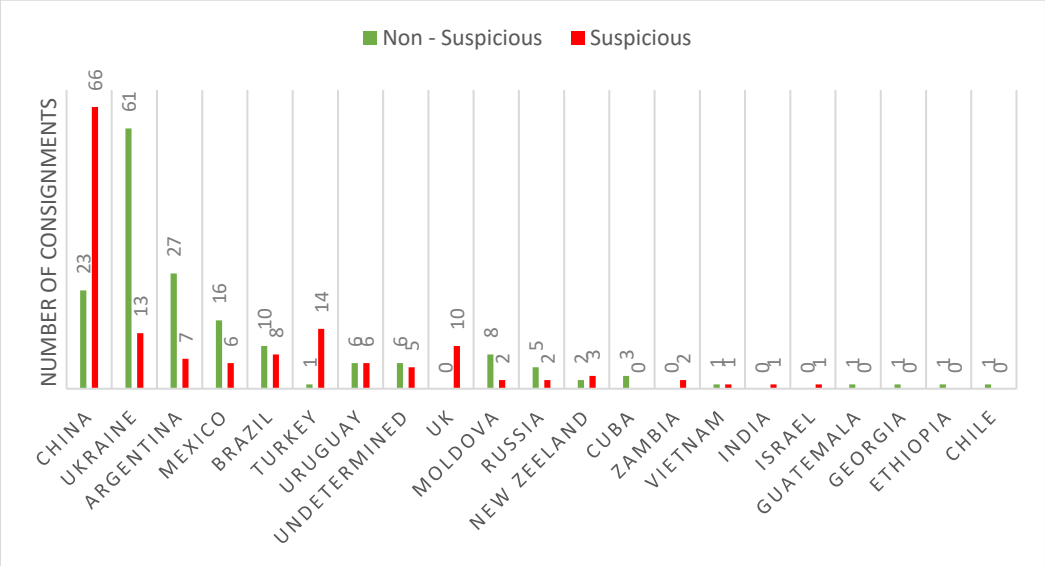


Figure 4. Geographical origin of suspicious honey consignments.

4. Conclusions

The EU coordinated action initiated by the European Commission confirmed that a substantial portion of honey imported into the EU is suspicious of being adulterated. The analysis of 320 honey consignments sampled in fifteen EU Member States, Norway, and Switzerland indicated that about 46% (147 samples) may not have been compliant with the EU Honey Directive 2001/110/EC [20]. Despite honey's potential health benefits and its value on the market, adulteration of honey has become an issue. The EU relies heavily on imported honey since its own production does not meet the demand. The cost disparity between genuine honey and sugar syrups, along with the challenge of detecting the extension of honey with syrups, offer attractive fraud opportunities for dishonest business operators. It is essential to conduct thorough tests to verify the purity of honey and protect consumer health. The state-of-the-art analytical methods selected in this study have confirmed their capacity to detect the addition of sugar syrups in honeys, but so far, they are not yet validated among laboratories. Therefore, there is an urgent need to develop and standardize advanced and widely accepted testing methods. Given the complexity of honey composition, which is influenced by many abiotic and biotic factors, the great diversity of adulterants, the unknowns linked to indirect adulterations, and the capacity of fraudsters to circumvent improvements in honey controls, the use of several complementary analytical methods measuring multi-analytes is essential.

Such methods should improve the ability of regulatory laboratories to identify honey adulterated with sugar syrups that imitate the natural sugar composition of genuine honey. Implementing these improved testing techniques will act as a powerful preventive measure against fraudulent practices in the international honey market.

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Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

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