

Cite this: *Analyst*, 2025, **150**, 4070

# Production process defects of MiXi beverage detected by neutral desorption extractive electrospray ionization mass spectrometry†

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Process defects in the model production process of MiXi beverage, typical examples of extremely viscous food products, have been successfully detected using neutral desorption-extractive electrospray ionization mass spectrometry (ND-EESI-MS) without sample pretreatment. We employed statistical analysis to explore the correlation between process defects in production and metabolite ions in the fingerprints of MiXi samples with diverse qualities, which were acquired using ND-EESI-MS. Abnormal signals in the fingerprint profiles, such as the unexpected presence of heavy metal ions or pesticides, as well as changes in beneficial compounds like D-glucose, can indicate defects or issues encountered in the production process. Meanwhile, we quantitatively analysed the concentrations of heavy metals and pesticide residues in MiXi beverage, and the results showed a strong linear relationship ( $R^2 > 0.99$ ) between heavy metals and pesticide residues in the concentration range  $0.50\text{--}200.00\text{ }\mu\text{g L}^{-1}$ , with a limit of detection (LOD) of  $0.04\text{--}1.44\text{ }\mu\text{g L}^{-1}$  and limit of quantification (LOQ) of  $0.12\text{--}4.36\text{ }\mu\text{g L}^{-1}$ , with an RSD of  $0.39\text{--}3.66\%$ . Furthermore, in contrast to previous methods, concealed defects occurring in the manufacturing process, including raw material screening, soaking, and extraction, can be directly detected by terminal product analysis, showing that ND-EESI-MS was a promising tool for quality control in viscous beverages and tracing defects in the production process.

Received 14th March 2025

Accepted 6th June 2025

DOI: 10.1039/d5an00299k

rsc.li/analyst

## 1. Introduction

In the chemical and food industries, in general, a defect means any malfunction in the manufacturing line and/or abnormal conditions leading to unexpected properties for a product. It is important to address all defects before delivering the product to the client, to maintain the client relationship and avoid huge economic losses.<sup>1</sup> Usually, the physical con-

ditions of a production line are monitored online by instrumental methods, including infrared thermal imaging,<sup>2,3</sup> equivalent circuit analysis,<sup>4</sup> thin film characteristic analysis,<sup>5</sup> as well as optical, electrical, and other technologies. Advances in the field have been systematically reviewed elsewhere.<sup>6,7</sup> To mitigate the impact of process defects, inspection procedures are implemented at multiple stages throughout food production. Additionally, sampling inspections are conducted before the products leave the factory. However, if substandard quality is identified during this final sampling, it becomes difficult to trace the exact stage at which the issue occurred. Consequently, not only must the entire batch be discarded, but the staff must also re-evaluate each stage of the subsequent batch's production. This leads to substantial economic losses and imposes significant time and labor demands on the production process. To date, the quality of the product has normally been evaluated by chemical analytical methods, including gas chromatography (GC),<sup>8,9</sup> high performance liquid chromatography (HPLC),<sup>10–12</sup> liquid chromatography-mass spectrometry (LC-MS),<sup>13,14</sup> gas chromatography-mass spectrometry (GC-MS)<sup>15–17</sup> and stable carbon isotope ratio analysis (SCIRA).<sup>18,19</sup> Most of these methods require extensive preprocessing of the product, which may result in the loss of some

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†Electronic supplementary information (ESI) available: Additional data analysis results, including process flow chart, PLS-DA model, information of differential metabolites, tables of compounds identification, regression equations, LODs and LOQs of pesticide analytes. See DOI: <https://doi.org/10.1039/d5an00299k>

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sample information. Process defects alter the chemical composition of the product, giving rise to inconsistent chemical fingerprints of the final products. However, efforts have rarely been made to diagnose process defects by analyzing the chemical composition of the final product. Therefore, it is highly desirable to develop rapid and sensitive analytical methods for directly detecting potential process defects by analyzing the chemical composition of the final product.

To demonstrate, a typically viscous and atherosclerotic rice food named a MiXi beverage was selected as the sample, which normally contains Chinese herbal medicines, such as *Euryale ferox*, yam, lotus seed, and *Poria cocos*. As formulated, it is rich in nutrients resulting from the complex production processes (Fig. S1†).<sup>20</sup> The components of MiXi beverage are complex and diverse. They include proteins, carbohydrates, crude fibers, vitamins, and minerals that are essential to the human body. In addition, the beverage contains numerous bioactive substances, such as polysaccharides, triterpenoids, volatile oils, alkaloids, flavonoids, glycosides, steroids, and fatty acids, which are beneficial to the human body.<sup>21–23</sup> The sample processing protocols are complicated, and it takes hours for a single run, which is time-consuming and laborious. This causes a lot of inconvenience for quality testing of products. Therefore, there is an urgent need to develop a system that is easy to implement, simple to operate, time-efficient, and capable of preserving sample integrity for the rapid and accurate detection of defective production steps or corresponding factors in the MiXi beverage production process.

Extractive electrospray ionization mass spectrometry (EESI-MS) allows for the direct analysis of diverse and intricate sample matrices without the need for any prior sample preparation. In EESI-MS, the initial step involves charging the chosen reagent (such as methanol or water) using electrospray ionization (ESI) to effectively generate an ionic reagent plume. This plume subsequently interacts with the sample plume in the open air, encompassing a substantial volume of approximately 20 cm<sup>3</sup>. During this interaction, the energetic charge is transferred to the analyte molecules derived from the sample mixture. Afterward, the analytes undergo ionization through extraction and collision processes that take place during interactions between the two plume sprays. As a result, the ionized analytes were introduced into the mass spectrometer for subsequent analysis.<sup>24</sup> ND-EESI-MS is an advanced technique derived from EESI, achieved by introducing suitable neutral gas plumes like air or nitrogen to efficiently extract analytes from highly viscous samples (*e.g.* honey, cheese, or toothpaste).<sup>25–29</sup> However, the use of ND-EESI-MS technology in process tracking for commercialized products has not yet been reported. Therefore, this study will establish an ND-EESI-MS analysis technology for analyzing the chemical composition of the MiXi beverage. Changes in the chemical composition of the products suggest that there may be loopholes in the selection of raw materials and process production and may help explain the origin of these changes in the chemical composition of the products. The chemical association

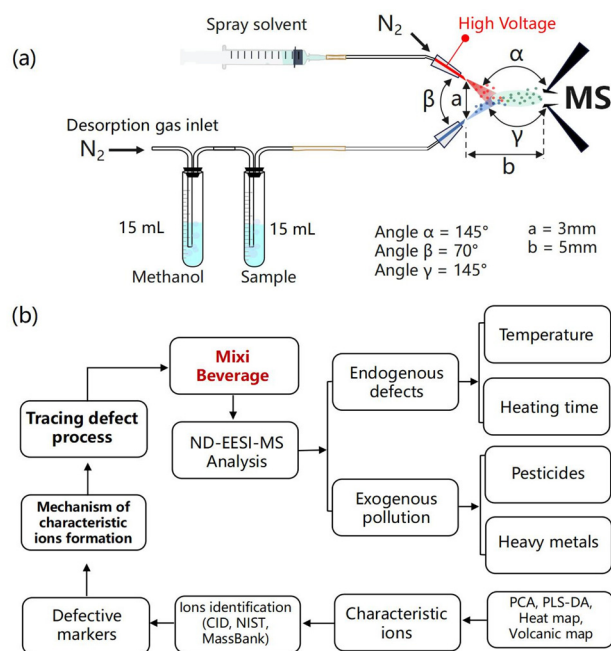


Fig. 1 Workflow diagram of the study design: (a) schematic diagram of ND-EESI-MS platform; (b) flowchart of ND-EESI-MS analysis.

with the raw material quality and production processes provides a powerful scientific tool for quality control and accurate diagnosis of defects in the MiXi beverage production process (Fig. 1).

## 2. Results and discussion

### 2.1. ND-EESI-MS fingerprint of MiXi beverage

The metabolomics approach is of great importance for identifying and associating chemical changes in food processing with the desired quality assessment of the final product.<sup>30</sup> Unlike targeted metabolomics for the main purpose of quantitative analysis, the untargeted approach is a more common choice for exploratory research, as it covers a wider range of molecules and is more suitable for identifying new metabolites.<sup>31</sup> To gain insight into the underlying relationships between molecules and process defects, we conducted molecular profiling of normal MiXi beverage samples and samples with manufactured defects. The total ion current (TIC) for MiXi beverage was visually analyzed by ND-EESI-MS in both positive ion and negative ion detection modes (Fig. 2). In the positive ion mode, a total of 1365 ions were detected, and 62 components were successfully identified by comparing them to the NIST database, MassBank database, and relevant literature. Likewise, in the negative ion mode, 584 ions were detected, and 17 components were identified.

Based on Table S1† the identified nutrients include various triterpenoids, such as tumulosic acid ( $[M + H]^+$ ,  $m/z$  487.4), 25-hydroxypachimic acid ( $[M + H]^+$ ,  $m/z$  545.4), dehydro-tumulosic acid ( $[M + H]^+$ ,  $m/z$  485.4), and pachimic acid

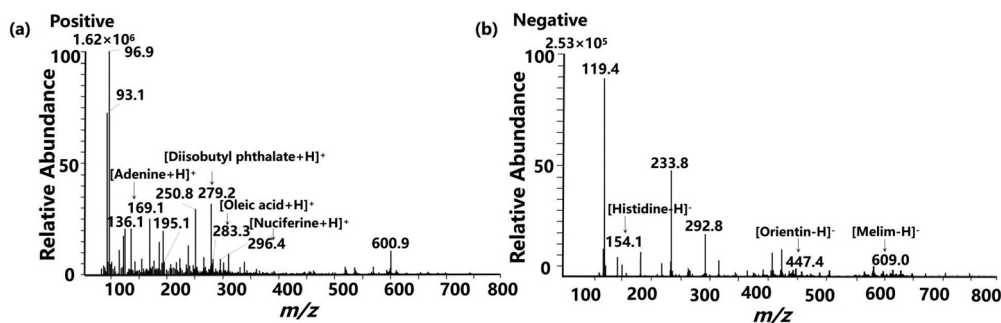


Fig. 2 Mass spectra of MiXi beverage acquired by ND-EESI-MS in the (a) positive and (b) negative ion modes.

$[M + H]^+$ ,  $m/z$  529.4). Additionally, nucleosides were detected, including adenosine ( $[M + H]^+$ ,  $m/z$  268.1), and adenine ( $[M + H]^+$ ,  $m/z$  136.1). The presence of alkaloids, such as neferine ( $[M + H]^+$ ,  $m/z$  625.0), tuduranine ( $[M + H]^+$ ,  $m/z$  298.1), and leonticine ( $[M + H]^+$ ,  $m/z$  328.2), was also observed. Moreover, flavonoids, such as orientin ( $[M - H]^-$ ,  $m/z$  447.0), isovitexin ( $[M - H]^-$ ,  $m/z$  431.0), and melim ( $[M - H]^-$ ,  $m/z$  609.0) were identified. Fatty acids, such as oleic acid ( $[M + H]^+$ ,  $m/z$  283.3), acetic acid ( $[M + K]^+$ ,  $m/z$  99.0), and tridecanoic acid ( $[M + Na]^+$ ,  $m/z$  235.2), were also observed. Finally, amino acids, including histidine ( $[M - H]^-$ ,  $m/z$  154.1), arginine ( $[M + H]^+$ ,  $m/z$  175.1), asparagic acid ( $[M + H]^+$ ,  $m/z$  134.1), and glutamic acid ( $[M + H]^+$ ,  $m/z$  148.1), were detected.

## 2.2. Evaluation of quality control of MiXi beverage production process

Extended or excessive heating can have detrimental effects on MiXi beverage, resulting in a decrease in volatile components and an increase in by-products, adversely affecting the overall quality. In addition, the duration of the soaking process plays a crucial role in the dissolution of water-soluble active ingredients. Coarse crushing of medicinal materials leads to a decrease in the content of active ingredients. Our findings indicate that relying solely on sensory methods to detect subpar MiXi beverage after subjecting them to varying treatments (heating at 50 °C for 1 h, heating at 100 °C for 1 h, heating at 100 °C for 6 h, and diluting with water thrice) may be insufficient. Accurately identifying defective products and localizing the specific production process responsible for the defect by detecting unique markers in the terminal product, represent valuable research.

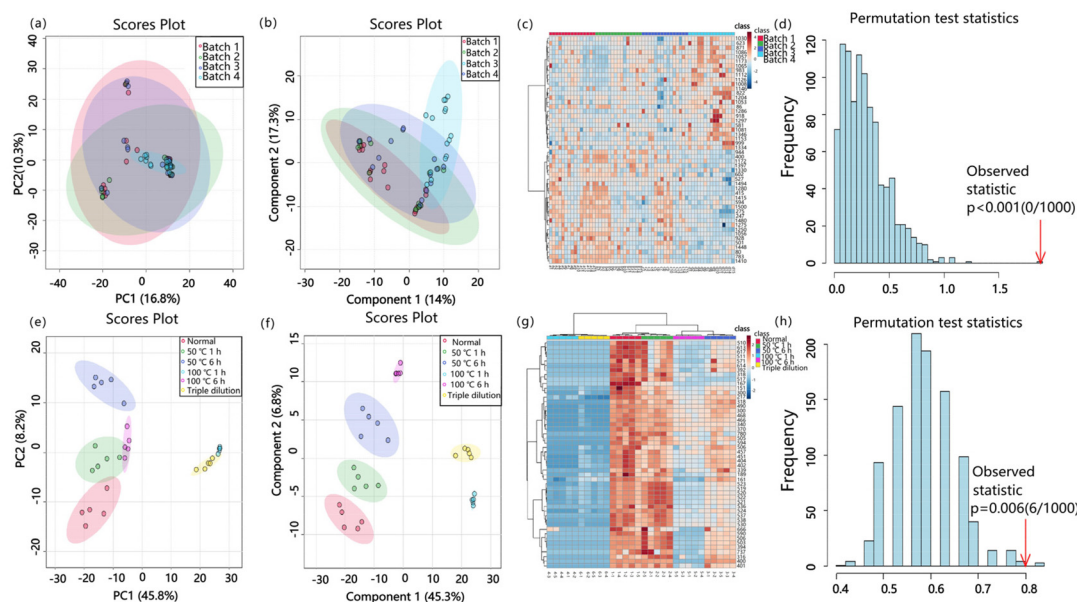
ND-EESI-MS was utilized to perform untargeted metabolomics analysis on both qualified normal MiXi beverage and five kinds of artificially prepared samples. Four batches of normal MiXi beverage cannot be distinguished in either the principal components analysis (PCA) model (an unsupervised statistical method, as depicted in Fig. 3a) or the PLS-DA model (Fig. 3b). The heatmap also showed that the signal intensities of the top 50 ions among the four groups of samples had no significant differences (Fig. 3c). In contrast, the molecular differences among the normal MiXi and five kinds of artificially prepared samples were readily visible in the PCA score plot (Fig. 3e),

demonstrating clear separation between the groups. After conducting a multivariate analysis, 50 critical metabolites ( $p < 0.05$ ) that significantly differentiated the six groups were observed (Fig. 3f and g).

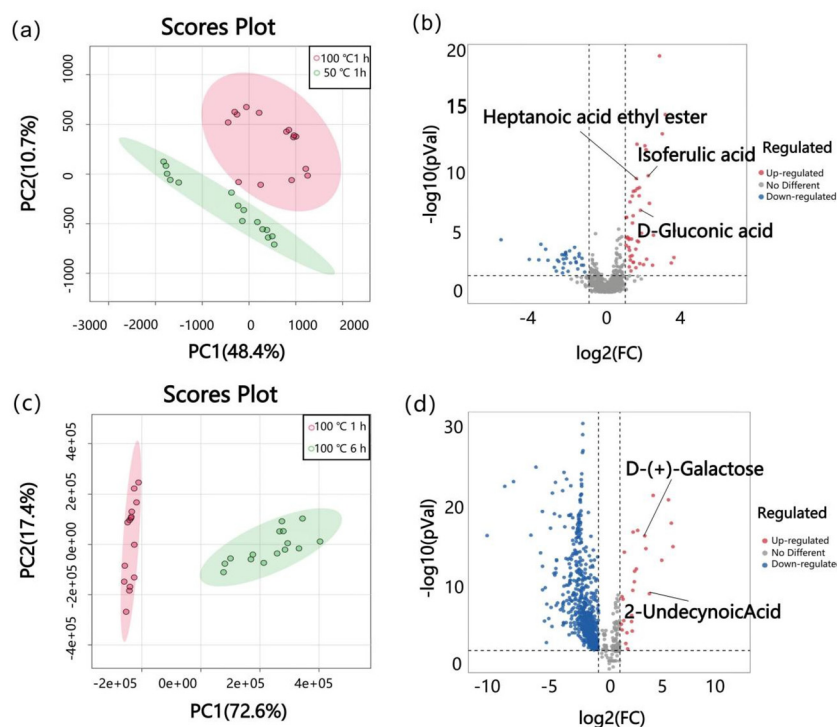
We thoroughly evaluated the classification ability of the PLS-DA model by analyzing three crucial model parameters:  $R^2X$  (explained variation in the  $X$  matrix),  $R^2Y$  (explained variation in the  $Y$  matrix), and  $Q^2Y$  (the predictive ability of the model). As depicted in Fig. S2a,† the  $Q^2$  value was 0.18182, indicating that the model lacked classification ability and was invalid. However, Fig. S2b† showed that the PLS-DA model was efficient in classifying the normal and defective groups with a  $Q^2$  value greater than 0.5. Moreover, no signs of overfitting were observed in Fig. 3d or h ( $p < 0.05$ ), indicating that the model is robust and capable of accurate classification.

## 2.3. The effect of extraction temperature and time

In the course of producing MiXi beverage, both the extraction and concentration process are carried out at temperatures between 40 and 60 °C.<sup>20,32</sup> Employing a low-temperature extraction and concentration process guarantees the stability of the active components, as well as facilitating their full dissolution without the risk of decomposition. We investigated the effect of extraction temperature and time on the quality of MiXi beverage. To simulate and validate the effect of extraction temperature in the production process of MiXi beverage, we heated a normal commercial MiXi beverage soup at 50 °C for 1 h. However, we found no significant difference compared to normal MiXi beverage, as shown in Fig. S3a.† Despite conducting statistical analysis on samples subjected to heating at 50 °C for 1 h and 6 h, no obvious difference was found (Fig. S3b†). This observation reflects the suitability of the extraction temperature and time (30–40 min) used in the production of commercial MiXi beverage, ensuring the stability and consistency of the quality of the final product. Likewise, we conducted an experiment where the normal MiXi beverage were heated at 100 °C for 1 h, and we then compared the mass spectra with those of the normal MiXi beverage. As a result, we observed a significant distinction between the two groups, which was further supported by the heatmap analysis, revealing 46 ions with significant changes in their intensity induced by the high extraction temperature (Fig. 4a and b). Among



**Fig. 3** ND-EESI-MS and metabolomics analysis of MiXi beverage: (a) PCA score plot of different batches of normal MiXi beverage; (b) PLS-DA score plot of different batches of normal MiXi beverage; (c) heatmap of four different batches of normal MiXi beverage; (d) permutation test statistics of different batches of normal MiXi beverage; (e) PCA score plot of MiXi beverage treated under different conditions; (f) PLS-DA score plot of MiXi beverage treated under different conditions; (g) heatmap of MiXi beverage treated under different conditions (treatment conditions: normal; heating at 50 °C for 1 h; heating at 50 °C for 6 h; heating at 100 °C for 1 h; heating at 100 °C for 6 h; triple dilution); (h) permutation test statistics of MiXi beverage treated under different conditions.



**Fig. 4** PCA and volcano map analysis of MiXi beverage treated with different heating conditions by ND-EESI-MS. Heating at 50 °C for 1 h and heating at 100 °C for 1 h (a) PCA score plot and (b) volcano plot; heating at 100 °C for 1 h and heating at 100 °C for 6 h (c) PCA score plot and (d) volcano plot.



them,  $m/z$  values, include 213, 282, 310, 137, 336, 525, 195, 231, 1395, 629, 196, 217, 124, 205, 127, 61, 681, 357, 161, 578, 160, 72, 203, 201, 148, 152, 519, 339, and 159, representing a set of unique marker ions that exhibit significant differences in intensity, highlighting the impact of the high extraction temperature on the quality of the final product. Some significant ions, such as isoferulic acid ( $m/z$  195), heptanoic acid ethyl ester ( $m/z$  159), and D-glucose acid ( $m/z$  196), which can be used as marker ions for referring to defects induced by high-temperature extraction, were identified using MS/MS experiments (Fig. 5).

A comparison between MiXi beverage heated at 100 °C for 1 h and those heated for 6 h at the same temperature revealed the presence of 26 different ions, of which 15 included  $m/z$  values of 137, 205, 163, 161, 169, 293, 555, 525, 556, 159, 201, 127, 145, 310 and 107 were selected as defect markers. The presence of these ions in the final product of MiXi beverage would indicate that the extraction or concentration process involved extended exposure to high-temperature, as depicted in Fig. 4c and d. Of particular note,  $m/z$  163 ( $[D-(+)-\text{galactose-H}_2\text{O} + \text{H}]^+$ ) and  $m/z$  205 ( $[2\text{-undecynoic acid} + \text{Na}]^+$ ), generated by the de- $\text{CH}_3$  of methyl 2-undecynoate after long-term high-temperature heating, were identified (Fig. 5).

#### 2.4. The effect of soaking time

Furthermore, notable variations were observed between the normal group and the triple dilution group, in which  $m/z$  values of 230, 229, 173, 151, 184, 100, 201, 142, and 232 exhibited significant changes following the triple dilution (Fig. S3c†), indicating a significant decrease in the concentration of key components. This suggests that the soaking time was likely too short, resulting in a low rate of dissolution of active ingredients and that inadequate homogenization may also have contributed to a reduced rate of dissolution. Another

potential cause of this defect may have been excessive water usage during the mixing process.

#### 2.5. Exogenous pesticide residue pollution

Pesticides are synthetic chemicals that exert significant biological effects and pose a substantial risk to both human health and the environment. Long-term exposure to and excess intake of these chemicals have been linked to various health hazards, including cancer, teratogenesis, and mutagenesis, as well as the potential transmission of viruses affecting the liver, kidneys, reproductive system, and nervous system.<sup>33,34</sup> Therefore, countries worldwide have implemented stringent regulations regarding pesticide residue levels in both food and the environment. China's recently updated national food safety standard, GB 2763-2021, outlines specific residue limits for 34 pesticides and their metabolites in rice and Chinese medicines.<sup>35,36</sup> ND-EESI-MS is advantageous for detecting trace compounds due to its ability to achieve low limits of detection (LODs).<sup>27,37,38</sup> Thus, we employed the ND-EESI-MS method to detect four pesticides and their metabolites in terminal samples of MiXi beverage to trace the safety of the raw materials. The LODs of MiXi samples spiked with diazinon,  $\beta$ -endosulfan, carbendazim, and methyl parathion, calculated using signal-to-noise ratios (S/N) of 3.3, were found to be 0.04–0.43  $\mu\text{g L}^{-1}$ . The LOQs estimated as the analyte concentration producing S/N of ten were 0.12–1.30  $\mu\text{g L}^{-1}$  (Table S2†). Moreover, the standard working curves for the four pesticide samples were constructed, and the linearities of the present method were evaluated. The correlation coefficients ( $r$ ) for these curves ranged from 0.9933 to 0.9982, indicating a strong linear relationship between signal intensity and concentration. The correlation coefficient of diazinon is 0.9982, the highest among all analytes. Simultaneously, we compared our method with the conventional detection method LC-MS, as shown in Table S3.† The relative standard

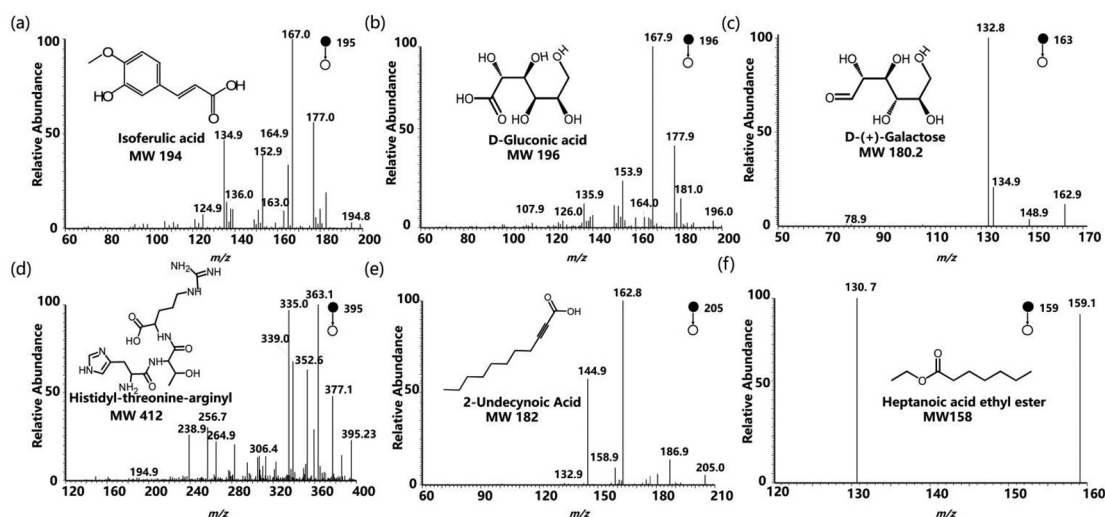


Fig. 5 ND-EESI-MS spectra of differential ions in MiXi beverage treated with different heating conditions: (a) isoferulic acid; (b) D-gluconic acid; (c) D-(+)-galactose; (d) histidine–threonine–arginine; (e) 2-undecynoic acid; (f) heptanoic acid ethyl ester.

deviations (RSDs) of LC-MS ranged from 1.64% to 6.43%. The results indicate that the method developed in this study exhibits better and more stable repeatability than LC-MS. This improvement may be attributed to the multiple sample pre-treatment steps in LC-MS, which introduce additional errors. Beyond the advantage of repeatability, LC-MS requires at least 10 h from sample processing to obtaining final results, whereas the entire ND-EESI-MS process takes only 1 min, significantly reducing analysis time and improving experimental efficiency.

We analyzed the differential ions associated with changes in rice quality resulting from pesticide contamination. Compared to normal MiXi samples, we observed characteristic ions indicative of pesticide residues, in addition to quasi-molecular ions. Distinctive ion signatures were identified for each pesticide *via* differential ion analysis (Fig. S4†). Diazinon exhibited characteristic ions at  $m/z$  93, 94, 149, and 311, with 311 serving as its diagnostic marker.  $\alpha$ -Endosulfan displayed specific ions at  $m/z$  121, 125, 135, 143, 153, 157, and 251, while  $\beta$ -endosulfan was distinguished by  $m/z$  108, 134, 227, 117, and 228. Carbendazim showed ions at  $m/z$  107, 122, 150, 177, and 199, and bifenthrin was uniquely marked by  $m/z$  133. Methyl parathion demonstrated specificity through its characteristic ions at  $m/z$  87, 147, and 310. Notably, shared ions ( $m/z$  93, 94, 105, 107, 119, and 149) were observed across multiple analytes. The identification of these differential ions was accomplished by using secondary mass spectra, as depicted in Fig. S5.†

## 2.6. Heavy metal pollution

Medicinal plants are susceptible to the presence of heavy metals, including lead (Pb), cadmium (Cd), and mercury (Hg), and detecting their presence is of the utmost importance. Notably, the levels of heavy metal content can differ significantly across various plant species, geographical regions, growth stages, and specific parts used for medicinal purposes.<sup>39</sup> Simultaneously, the proximity of food processing plants to regions experiencing significant environmental pollution, combined with inadequate water quality standards, can result in the accumulation of excessive levels of heavy metals. Therefore, screening out the characteristic ions of heavy metals through ND-EESI-MS analysis in terminal samples of MiXi beverage will be helpful for tracing heavy metal pollution in raw materials in MiXi processing, and guaranteeing safety, quality, and efficacy in food production.

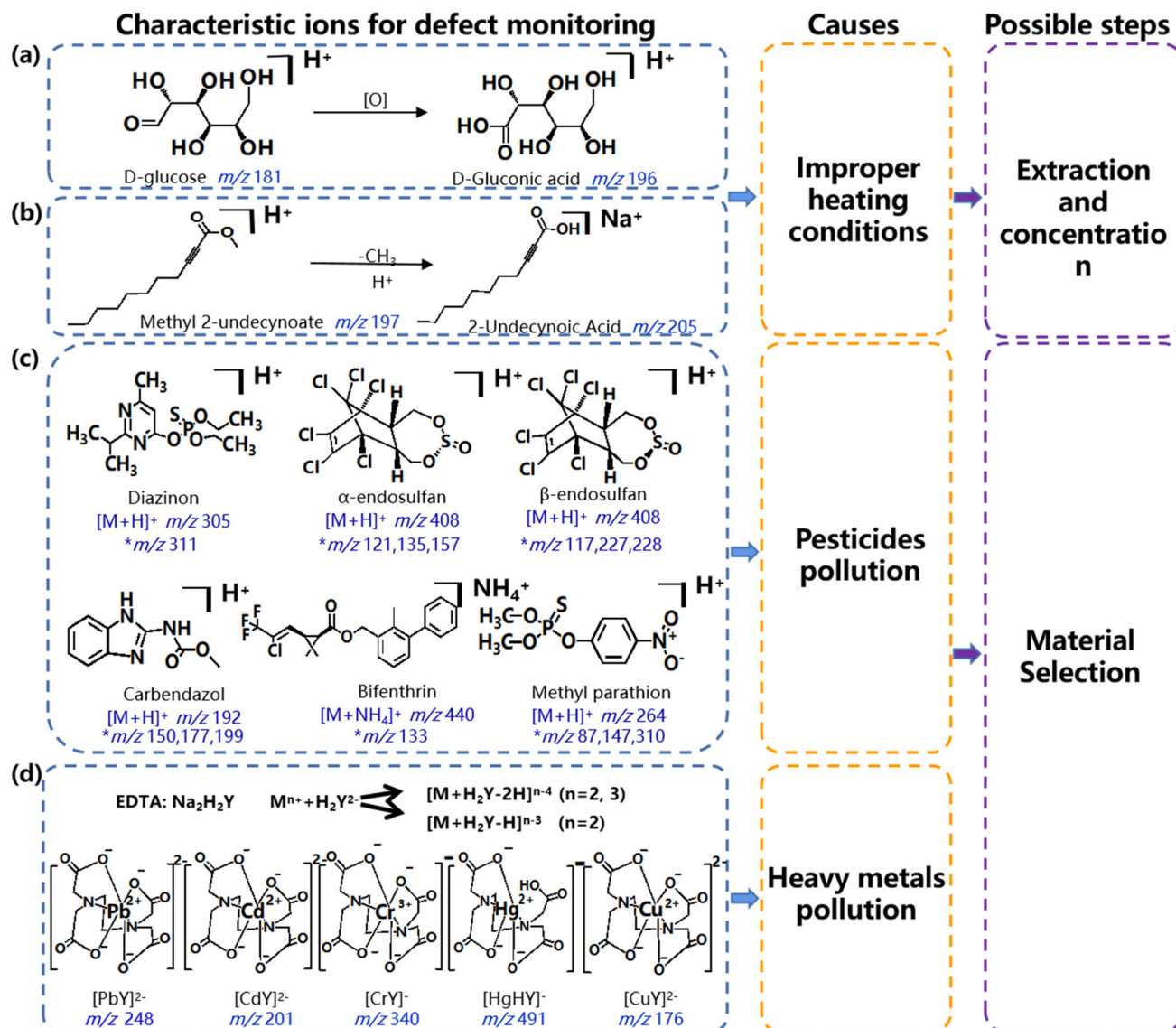
However, heavy metal ions are commonly found in the form of inorganic salts, and these salts do not readily evaporate under the typical conditions employed in organic mass spectrometry analysis. Consequently, they are unable to enter the mass spectrometer for ionization and subsequent analysis, presenting a notable obstacle in the detection of heavy metal ions using organic mass spectrometry. According to our previous work,<sup>40</sup> we utilize EDTA as an extraction reagent, because its exceptional chelating capability facilitates the formation of complexes with more than 90% of metal ions. These complex ions facilitated the rapid detection of heavy metal ions using organic mass spectrometry. We performed quantitative ana-

lysis experiments in the negative ion mode. Table S4† summarizes the metals, limit of detection (LOD), and limit of quantification (LOQ) and national standard for drinking water (GB 5749-2022). Complexes of heavy metal ions with EDTA, such as  $[\text{PbY}]^{2-}$  ( $m/z$  248),  $[\text{CdY}]^{2-}$  ( $m/z$  201),  $[\text{CrY}]^{-}$  ( $m/z$  340),  $[\text{HgHY}]^{-}$  ( $m/z$  491), and  $[\text{CuY}]^{2-}$  ( $m/z$  176) were detected. The results unequivocally demonstrate that the LOD values for  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Cu}^{2+}$  in MiXi were consistently below the detection limits prescribed by the national standard for drinking water (GB 5749-2022). Among these metals,  $\text{Cd}^{2+}$  exhibited remarkable sensitivity, while  $\text{Cu}^{2+}$  showed relatively weaker sensitivity in detection compared to their respective national standard limits (Table S4†). When the proposed heavy metals in the terminal MiXi products were detected to exceed the concentration allowed in the national standard, this suggested existing defects in the processing steps, such as the introduction of sub-standard raw materials, exceeding the limits of heavy metals in raw material screening or during soaking, grinding, and extraction processes, where contaminated water was utilized.

The analytical performance of inductively coupled plasma mass spectrometry (ICP-MS) and the proposed ND-EESI-MS method for the determination of heavy metals in MiXi beverages was systematically evaluated, as summarized in Table S5.† The ICP-MS method exhibited relative standard deviations (RSDs) ranging from 0.85% to 38.49% and recoveries of 68.0–123.1%. In contrast, the ND-EESI-MS protocol demonstrates superior repeatability and accuracy compared to ICP-MS. The observed limitations of ICP-MS may arise from two critical factors. Digestion-mediated sample preparation introduces inherent variability due to incomplete recovery of analytes and potential adsorption of analytes onto the inner walls of the sample introduction system, thereby introducing systematic errors. The protracted workflow of ICP-MS, requiring a minimum of 13 h from sample pretreatment to data acquisition, exacerbates the risks of contamination and analyte degradation. Conversely, ND-EESI-MS eliminates digestion requirements and completes the entire analytical process within 1 min, significantly minimizing operational uncertainties.

Further, through a comparative analysis of the mass spectra between the normal MiXi samples and those supplemented with heavy metal ions, rigorous statistical analysis demonstrated the discernible influence of heavy metal ions on the quality of the MiXi beverage (Fig. S6†). When  $\text{Pb}^{2+}$  appears in rice samples, the differential ions observed are  $m/z$  146, 165, 313, 150, 164, 145, 149, 684, 182, 158, 438, and 268. The introduction of  $\text{Cd}^{2+}$  generates differential ions at  $m/z$  93, 155, 61, 97, 165, 63, 123, 91, 125, 206, 145, 261, 125, and 154. The presence of  $\text{Cr}^{3+}$  leads to the observation of differential ions at  $m/z$  292, 344, and 62.  $\text{Hg}^{2+}$  results in the detection of differential ions at  $m/z$  313 and 314. Finally, the addition of  $\text{Cu}^{2+}$  gives rise to differential ions observed at  $m/z$  291, 292, 314, 313, 344, 164, and 62 in the MiXi samples.

The detection of specific product ions, such as D-glucuronic acid and 2-undecynoic acid, in MiXi beverage suggests the existence of process defects that lead to non-compliant



**Fig. 6** Mechanism of formation of characteristic ions and defects process tracing: (a) heating at 50 °C for 1 h and heating at 100 °C for 1 h; (b) heating at 100 °C for 1 h and heating at 100 °C for 6 h; (c) differential ions before and after adding pesticides; (d) differential ions before and after adding heavy metals. \*Characteristic ions.

product quality. These defects can be attributed to the use of inappropriate heating conditions during the extraction and concentration stages of the manufacturing process (Fig. 6a and b). The detected signals of pesticide residues, along with their associated metabolite ions and complex ions of heavy metals in the MiXi product, indicate the manifestation of process defects during the stage of raw material selection (Fig. 6c and d).

### 3. Materials and methods

#### 3.1. Samples and chemicals

Different batches of MiXi beverage (batches 20220506; 20220507; 20220608; 20220816) produced by Jiangzhong

Food Therapy Technology Co Ltd (in Nanchang, Jiangxi Province, China) were purchased from local markets and official flagship shops. Organophosphorus pesticides (HPLC grade purity, 96.8–99.5%), including diazinon,  $\alpha$ -endosulfan,  $\beta$ -endosulfan, bifenthrin, methyl parathion and carbendazim were purchased from Aladdin (Shanghai, China). Heavy reagents (AR grade), including lead acetate, cadmium chloride, chromium nitrate, mercury nitrate, and copper nitrate, were purchased from Aladdin (Shanghai, China). Methanol, acetone and acetic acid (all LC-MS grade) were provided by ROE Scientific Inc. (Newark, U.S.A). Disodium ethylene diamine tetraacetate (EDTA) was purchased from Aladdin (Shanghai, China). The deionized water used for the experiments was provided by a Hetai Smart-DUVF water purifier (Shanghai, China).

### 3.2. Study design

An untargeted metabolomics profiling method for discovering food production process defects was designed by detecting biomarkers of different quality samples of MiXi beverage based on ND-EESI-MS. The standard MiXi production process contains several steps: material selection, batch feeding, soaking, homogenization, extraction and concentration, and additive addition. Initial research mainly analyzed the influence of exogenous (heavy metals and organophosphorus pesticide pollution) and endogenous substances on the quality of MiXi beverages from the end product. This paper focused mainly on two exogenous and three other factors of improper soaking time, control of temperature and time.

In brief, we prepared MiXi samples with defects resulting from insufficient soaking time by diluting normal batches of MiXi with ultrapure water three times. Additionally, MiXi samples with defects resulting from improper control of temperature and time were prepared by individually adjusting the heating temperature and time of normal batches of MiXi. Heavy metal and organophosphorus pesticide pollution samples ( $1 \text{ ng L}^{-1}$  to  $5 \text{ mg L}^{-1}$ ) were produced by spiking normal batches of MiXi samples with a series of pollutant standard solutions. A summary of a representative artificially prepared sample is provided in Table S6.† A total of 67 samples, including four batches of normal MiXi samples and seven kinds of prepared MiXi samples, were analyzed using ND-EESI-MS analysis.

### 3.3. Analysis of pesticide residue and heavy metals in MiXi beverages by ND-EESI-MS

**3.3.1. Mass spectrometry conditions of ND-EESI-MS.** The ND-EESI-MS experiments were carried out with an in-house developed neutral desorption electrospray ion source coupled with a linear trap (LTQ) mass spectrometer (Thermo Scientific, Finnigan, U.S.A.), which was equipped with the Xcalibur data-processing system.

The voltages used for spray ionization were 4 kV and 3.5 kV under positive ion and negative ion detection modes, respectively. The capillary temperature, capillary voltage and tube lens voltage of the LTQ-MS were maintained at  $200^\circ\text{C}$ , 1 V and 10 V, respectively. A gentle nitrogen sheath gas stream ( $0.1 \text{ MPa}$ ) was blown into the methanol solution to form a mixture gas of nitrogen/methanol, which continuously brings the ingredients in the MiXi beverage out into the ionization region for primary ion production. Subsequently, the spray solvent, a mixture of methanol–water–glacial acetic acid ( $47.5:47.5:5$ , v/v) was passed through the quartz capillary at a flow rate of  $5 \mu\text{L min}^{-1}$ . Under the action of the electric field (spray solvent) and with the help of the nitrogen sheath gas stream ( $0.8 \text{ MPa}$ ), the spray solvent first obtains energy and forms charged reagent droplets. Conditional optimization results are shown in Fig. S7.† They are carriers of charge and energy and converge with analytes in three-dimensional space for ion exchange and ionization. The analyte in the sample is then extracted into the charged reagent droplets, followed by desolvation to generate

ions of the analyte for subsequent mass spectrometry analysis. The width of the parent ion isolation was  $1.0\text{--}1.5 \text{ Da}$ , and the collision energy was 15% to 35% in collision induced dissociation (CID) experiments. Other conditions were optimized by the system itself. The mass spectrometry scan range was  $m/z$  50–1000. The principle schematic diagram of ND-EESI-MS is shown in Fig. 1a.

**3.3.2. Method validation.** Following optimization of instrumental parameters, the analytical method for detecting four pesticide residues (diazinon,  $\beta$ -endosulfan, carbendazim, methyl parathion), and five heavy metals (Pb, Cd, Cr, Hg, Cu) in MiXi beverage was systematically validated, including assessments of linearity, sensitivity, precision, and accuracy.

**3.3.2.1. Linearity range and sensitivity.** Linearity refers to the proportional relationship between analyte concentration and experimental response within the designated range. Mixed standard working solutions were prepared by serially diluting stock solutions of each target analyte to six concentration levels (Tables S12 and S13†). Each concentration was analyzed in triplicate using ND-EESI-MS (Section 3.3.1), with secondary characteristic fragment ions selected as quantitative ions. Calibration curves were plotted by correlating the mean signal intensity (y-axis) with analyte concentration (x-axis), and linearity was evaluated *via* the correlation coefficient ( $R^2$ ) (Tables S2 and S4†).

Method sensitivity was evaluated by determining the LOD and LOQ using the following relationships:

$$\text{LOD} = 3.3\sigma/b, \text{LOQ} = 10\sigma/b$$

where  $\sigma$  denotes the standard deviation of baseline noise derived from nine blank measurements, and  $b$  represents the slope of the calibration curve.

**3.3.2.2. Repeatability and accuracy.** Repeatability, reflecting the consistency of results under identical conditions, was quantified *via* RSD. For pesticide residues and heavy metals, three concentration levels (low, medium, high) of spiked MiXi beverage blank samples were independently prepared in triplicate (Tables S14 and S15†). Each level was analyzed following Section 3.3.1, and RSD values were calculated from triplicate measurements to assess method repeatability.

Accuracy, expressed as spike recovery (%), was evaluated by spiking blank MiXi beverage samples with target analytes at three concentration levels. Triplicate spiked samples and three unspiked blanks were processed and analyzed as per Section 3.3.1. Recovery rates were calculated by comparing measured concentrations with theoretical spiked values (Tables S7 and S8†).

**3.3.3. Matrix effect evaluation.** Matrix effects (ME), defined as the ratio of the analyte response in the sample matrix ( $B$ ) to that in pure solvent ( $A$ ) ( $\text{ME} = B/A$ ), may induce signal enhancement or suppression. An ME ratio within the range  $0.9\text{--}1.1$  signifies negligible matrix interference. The results demonstrated negligible ME for the three pesticide residues, except for diazinon, which showed a weak matrix effect (Table S2†), and for the five heavy metals, which showed a weak matrix effect (Table S4†). This selective interference underscores the neces-



sity of matrix-matched calibration for diazinon and mercury to ensure quantitative accuracy.

**3.3.4. Statistical analysis.** The differences between MiXi beverage samples were visualized using mass spectral raw data formatted into .xls files and analyzed with the commercially available website MetaboAnalyst (<https://www.metaboanalyst.ca>). We aligned and averaged the responses of each ion signal from 30–40 mass spectral data files, which were collected over 10 s, to obtain an average signal value for each ion and finally form a complete mass spectral dataset for one sample. The independent variables in the analysis were the  $m/z$  values, with the signal intensities acting as dependent variables for all the text data. The data matrix was automatically loaded into STATISTIC software as a standard feature of the mass data file. Subsequently, the data were normalized and subjected to PCA, partial least squares discriminant analysis (PLS-DA), volcano maps, and heatmap analyses. Any features with more than 20% missing values were excluded from the data matrix. Multivariate statistical analysis was performed on the data matrix to obtain  $p$  values, VIP values, and fold change (FC) values. Potential differential metabolites were identified by applying a criterion of  $p < 0.05$ ,  $VIP > 1$ , and  $FC > 2$ . Those with matching scores below 70 and exogenous differential metabolites were excluded. Afterwards, the molecular structures were identified by comparison with NIST, MASSBANK, HMDB and other databases and published literature.

### 3.4. Analysis of pesticide residue in MiXi beverage by LC-MS

**3.4.1. Sample preparation for MiXi beverage.** A 5 mL aliquot of the diluted MiXi beverage was accurately pipetted into a 50 mL centrifuge tube. Subsequently, 10 mL of acetonitrile, a QuEChERS extraction salt packet (6 g of anhydrous magnesium sulfate, 0.904 g of anhydrous sodium acetate), and one ceramic homogenizing pellet were added. The tube was tightly sealed and vigorously shaken by hand for 1 min, followed by 10 min of vortex-mixing using a mechanical shaker. The mixture was then centrifuged at 4200 rpm for 5 min. From the resulting supernatant, 3 mL was aspirated and transferred into a plastic centrifuge tube preloaded with a dispersive clean-up sorbent mixture (0.9 g of anhydrous magnesium sulfate, 150 mg of primary secondary amine, and 15 mg of graphitized carbon black). After manual shaking to homogenize the mixture, the tube was vortex-mixed for 5 min and centrifuged again at 4200 rpm for 5 min. The final supernatant was filtered through a polyethersulfone (PES) 0.22  $\mu\text{m}$  syringe filter prior to instrumental analysis.

**3.4.2. Preparation of standard solutions for LC-MS experiments.** Accurate volumes of four pesticide standard solutions (diazinon: 100  $\mu\text{g mL}^{-1}$ ,  $\beta$ -endosulfan: 100  $\mu\text{g mL}^{-1}$ , carbendazim: 10  $\mu\text{g mL}^{-1}$ , methyl parathion: 10  $\mu\text{g mL}^{-1}$ ) were precisely pipetted and diluted to volume with acetonitrile to prepare a mixed intermediate standard stock solution. This stock solution was serially diluted with acetonitrile to generate a gradient concentration series of working standards at 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2  $\text{mg L}^{-1}$ . All working solutions were freshly prepared prior to analysis to ensure optimal stability and mini-

mize analyte degradation. The peak area of the target component was taken as the vertical coordinate, with the mass concentration of the corresponding target component in the on-board standby solution as the horizontal coordinate to draw the standard curve.

**3.4.3. Chromatographic and mass spectrometric conditions.** Chromatographic separation was achieved using a Waters  $\text{C}_{18}$  column (100 mm  $\times$  2.1 mm, 1.7  $\mu\text{m}$  particle size) maintained at 30  $^{\circ}\text{C}$ . The mobile phase comprised (A) an aqueous solution of 2 mmol  $\text{L}^{-1}$  ammonium acetate and 0.01% (v/v) formic acid, and (B) acetonitrile, with a gradient elution program outlined in Table S9.† The flow rate was set to 0.3  $\text{mL min}^{-1}$ , and the injection volume was 2  $\mu\text{L}$ . The mass spectrometer was equipped with an ESI source operated in dual-polarity scanning mode, simultaneously acquiring data in both positive ( $\text{ESI}^{+}$ ) and negative ( $\text{ESI}^{-}$ ) ion modes. Detection was performed using multiple reaction monitoring (MRM). Key operating parameters were configured as follows: the interface voltage was 3.0 kV, the interface temperature was 300  $^{\circ}\text{C}$ , the desolvation line temperature was 250  $^{\circ}\text{C}$ , and the heating module temperature was 400  $^{\circ}\text{C}$ . Gas flow settings included a nebulizing gas flow rate of 3  $\text{L min}^{-1}$ , a drying gas flow rate of 10  $\text{L min}^{-1}$  and a heating gas flow rate of 9  $\text{L min}^{-1}$ . The MRM parameters for the determination of the four pesticide residues are shown in Table S10.†

**3.4.4. Repeatability and accuracy.** Three distinct concentrations of pesticide residue standard solutions (0.11, 0.55, and 2.2  $\text{mg L}^{-1}$ ) were prepared. For each spiked sample, 10 mL of the standard solution was added to 100 mL of blank MiXi beverage matrix samples, yielding target concentrations of 0.01, 0.05, and 0.2  $\text{mg L}^{-1}$ . The spiked samples were analyzed according to the procedure outlined in Section 3.4.3. Each concentration level was prepared in triplicate ( $n = 3$ ), and blank samples were similarly extracted three times ( $n = 3$ ) as controls. For reproducibility evaluation, three replicate samples per concentration were analyzed, with duplicate measurements performed for each replicate. Chromatographic data for all spiked samples were processed by extracting integrated values from five predefined regions of interest (ROIs) within the chromatograms: baseline within the retention time window, pre-peak segment, peak apex segment, post-peak segment, and total peak area. For each concentration level, the mean response value, standard deviation (SD), and RSD were calculated across these ROIs. Recovery rates were determined by comparing measured concentrations to theoretical spiked values.

### 3.5. Analysis of heavy metals in MiXi beverage by ICP-MS

**3.5.1. Microwave digestion of MiXi beverage.** A 1 mL aliquot of the liquid sample was accurately pipetted into a microwave digestion vessel, followed by the addition of 5 mL of nitric acid. The mixture was loosely capped and allowed to pre-digest for 1 h or overnight. The vessel was then tightly sealed and subjected to microwave digestion following the manufacturer's standard protocol (see Table S11† for digestion parameters). After cooling to ambient temperature, the vessel was cautiously opened to slowly vent residual pressure. The

inner lid was rinsed with a minimal volume of ultrapure water, and the digestate was transferred to a temperature-controlled hot plate or ultrasonic bath for post-digestion treatment: heating at 100 °C for 30 min. The digested solution was subsequently adjusted to a final volume of 25 mL with ultrapure water, homogenized, and stored for analysis. Parallel reagent blank digestions were processed identically to account for background interference.

**3.5.2. ICP-MS operational parameters.** Helium collision reaction cell mode was used to optimise the instrument mass axis, resolution, sensitivity, double charge and oxides with tuning solution. The instrument parameters were set as follows: argon as carrier gas, helium as collision gas, plasma gas flow rate of 15.0 L min<sup>-1</sup>, sampling depth of 8.0 mm, carrier gas volumetric flow rate of 1.17 L min<sup>-1</sup>, Barblinton nebuliser, quartz dual-channel nebulising chamber, nebulising chamber temperature of 2 °C, plasma RF power of 1.3 kW, collision mode, helium flow rate of 5.0 mL min<sup>-1</sup>, peristaltic pump speed of 30 rpm, 3 measurement points, analysis time of 0.1 s, three repetitions, and full quantitative analysis mode.

**3.5.3. Preparation of standard solutions.** Appropriate volumes of Cu, Cr, Cd, and Pb standard stock solutions were pipetted, and diluted with 2% nitric acid solution to prepare mixed solutions containing Cr, Cd, and Pb at concentrations of 0, 1, 2, 5, 8, and 10 µg L<sup>-1</sup>, and Cu at 0, 10, 20, 50, 80, and 100 µg L<sup>-1</sup>. Separately, an appropriate volume of Hg standard stock solution was accurately pipetted and diluted with 2% nitric acid solution to prepare Hg solutions at concentrations of 0, 0.1, 0.2, 0.5, 0.8, and 1.0 µg L<sup>-1</sup> (freshly prepared before use).

**3.5.4. Repeatability and accuracy.** To validate the precision and accuracy of the method, spiked recovery experiments were performed for target metals Cr, Cd, Pb (spiked concentrations: 1, 5, 10 µg L<sup>-1</sup>), Cu (10, 50, 100 µg L<sup>-1</sup>), and Hg (0.1, 0.5, 1.0 µg L<sup>-1</sup>). Pre-treated MiXi beverage samples ( $n = 3$  per concentration level) were spiked with target metal standard solutions at designated concentrations. The spiked samples were processed according to the method described in Section 3.5.1. Analysis was conducted using the optimized ICP-MS parameters in Section 3.5.2, with reagent blanks and unspiked control samples included. Recovery rates were calculated as the percentage of the measured concentration (after subtracting the background value) relative to the theoretical spiked concentration. Repeatability was evaluated based on the RSD of five replicate measurements at each spiking level.

## 4. Conclusion

In this study, the ND-EESI-MS technique was employed to analyze the mass spectra of various substances in MiXi beverage, demonstrating its potential for rapid, online determination of complex matrix samples. ND-EESI-MS has high sensitivity, rapid analysis and excellent specificity, which are common characteristics of detection methods (such as LC-MS or GC-MS). Unlike the other methods, ND-EESI-MS enables direct online analysis of differential ions in MiXi beverage

without the need for sample pretreatment. Through the identification of these distinctive ions, the technological defects within the production process of MiXi beverage can be deduced and traced back, providing valuable insights for raw material selection and production process monitoring to ensure beverage quality. The establishment and appropriate application of the ND-EESI-MS method can effectively monitor the quality of a MiXi beverage, and the proposed method showcases its remarkable efficacy and promising potential for wider implementation in food quality control and defect monitoring throughout the entire production process, solely by analyzing the terminal MiXi samples.

## Author contributions

Lingling Pan, Manman Qin, Lu Huang, Huiyu Xing and Ning Wang: performing data acquisition by ND-EESI-MS. Manman Qin, Lu Huang, Huiyu Xing, Tongtong Yang and Liyun Hu: conducting data preprocessing and statistical analysis. Rui Su: drafting the manuscript. Huanwen Chen: supervising the data interpretation. Rui Su had primary responsibility for the content of the manuscript. All authors provided critical input, revised, and approved the final version.

## Data availability

The authors declare that the data supporting the findings of this study are available within the paper. Should any data files be needed in any other format, they are available from the corresponding author upon reasonable request.

## Conflicts of interest

The authors declare no competing financial interest.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 82004005; 91959201), Jiangxi University of Chinese Medicine School-level Science and Technology Innovation Team Development Program (No. CXTD22005), High-level Talents of Chinese Medicine (No. 13030599) and Virtual Office for teaching and research (Xiao Zi [2022] No.41)

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