Journal of Analytical Science and Technology

RESEARCH ARTICLE Open Access

Quantitative analysis of galactose using LDI-TOF MS based on a TiO_2 nanowire chip

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Abstract

A novel method for quantifying galactose was developed to serve as a newborn screening test for galactosemia using laser desorption/ionization time-of-flight (LDI-TOF) mass spectrometry (MS) with a TiO_2 nanowire chip. Herein, phosphate citrate buffer, serum, and dried blood spot (DBS) were employed for the quantitative analysis of galactose. To quantitatively analyze galactose, its reduction potential was used to oxidize *o*-phenylene diamine (OPD) into 2,3-diaminophenazine (DA), which were both detected using LDI-TOF MS with a TiO_2 nanowire chip according to the concentration of galactose. The reproducibility and the interference of glucose were determined to demonstrate the applicability of this method. Moreover, mixtures of galactose, phenylalanine, and 17 α-OHP were analyzed to determine the interference induced by other biomarkers of metabolic disorders. The OPD oxidation of galactose was found to be selectively achieved under high-glucose conditions, similar to human blood, thereby showing good reproducibility. The intensities of the mass peaks of OPD and DA based on LDI-TOF MS with a TiO_2 nanowire chip were linearly correlated in the galactose concentration range of 57.2–220.0 μg/mL ($r^2 = 0.999$ and 0.950, respectively) for serum samples and 52.5–220.0 μg/mL ($r^2 = 0.993$ and 0.985, respectively) for DBS after methanol precipitation/extraction. The enzyme immunoassay and LDI-TOF MS analysis results were statistically analyzed, and a mixture of phenylalanine, 17 α-OHP, and galactose was simultaneously investigated quantitatively at the cutoff level.

Keywords: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, TiO_2 nanowire chip, Galactose, Reduction power, *o*-phenylene diamine, 2,3-diaminophenazine, Galactosemia

Introduction

Detection of galactose is important in many fields, including food science, medicine, and nutrition (Alm 1982; Nguyen et al. 2020; Rajendran and Irudayaraj 2002; Kaarj et al. 2020). The control of galactose level is extremely crucial as life-threatening symptoms can occur, particularly in newborns with undiagnosed galactosemia. High galactose concentration in the body may cause fatal galactosemia, which is manifested as feeding problem, cirrhosis, severe brain damage, jaundice, sepsis with *Escherichia coli*, cataract, and in females, premature ovarian insufficiency and several other complications (Levy and Hammersen 1978; Pyhtila et al. 2015).

Newborn screening tests have been employed to diagnose many types of fatal diseases; these tests involve the analysis of essential metabolites involved in their growth, such as amino and fatty acids (Naylor and Chace 1999; Paul 2008; Pitt 2010; Ryckman et al. 2013; Saudubray et al. 2002). In particular, newborn screening tests involving the analysis of amino and fatty acids have been extensively conducted using liquid chromatography with tandem mass spectrometry (LC–MS/MS) (Kaye et al. 2006; Lee 2006; Li and Tse 2010; Shim et al. 1999). Newborn screening program for galactosemia is generally accompanied by the detection of galactose metabolites (i.e., galactose and galactose-1-phosphate) in blood samples collected from heel capillary and DBS sample

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based on other test platforms such as laser-induced fluorescence and enzyme immunoassay (EIA) (Cheon et al. 2008; Freer et al. 2010; Park et al. 2003; Sohn 2015; Gao et al. 2021). The defined critical cutoff for total galactose is typically > 11 mg/dL (0.6 mmol/L) for the diagnosis of galactosemia (Cheon et al. 2008). However, highly specialized instruments are usually required for these techniques, in addition to intricate sample processing, which is influenced by high temperature, humidity, and duration between sampling and testing. Therefore, developing a simple, sensitive, single test platform for effective newborn screening tests is highly required for total and simultaneous analyses of the targeted metabolite repertoire, from small molecules (amino acids, fatty acids, and monosaccharides) to large proteins.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) provides rapid detection of biomolecules with high sensitivity and accuracy in a short detection time (Hillenkamp et al. 1991; Karas and Hillenkamp 1988; Jang et al. 2017). A small amount of sample is required for analysis and simultaneous identification can be achieved using MALDI-TOF MS, which makes MALDI-TOF MS a useful multiplex measurement platform for newborn screening. However, in small molecule analysis, MALDI-TOF MS has the major limitation of severe unreproducible organic matrix-related noise in the low *m/z* range (Guo et al. 2002; Wei et al. 1999). Recently, laser desorption/ionization time-of-flight (LDI-TOF) MS based on a TiO_2 nanowire chip was developed for the quantitative analysis of small molecules and minimization of the interference of mass peaks of organic matrices at a low *m/z* range of less than 1000 (Kim et al. 2014, 2016, 2020). In the LDI-TOF MS, inorganic matrices are used instead of organic matrices for the conventional MALDI-TOF MS. TiO_2 nanowire chip was reported to detect proteins with molecular weight as high as 20 kDa. Accordingly, this method could be applicable as a single test platform for newborn screening tests based on measurements of amino acids and proteins. However, among the biomarkers of target diseases, monosaccharides such as galactose are impossible to directly detect using LDI-TOF MS because of its low ionization efficiency caused by the structural properties of monosaccharides (Busse et al. 2006; Harvey 2015; Mechref et al. 2003; Pham et al. 2016). Until now, the measurement of amino acids for the newborn screening test has been carried out using LC–MS and the measurement of galactose has been separately performed using an enzyme assay. This work aimed to demonstrate the simultaneous measurement of amino acids and galactose using LDI-TOF MS. However, galactose is not easily ionized for LDI-MS, and the modification reaction needs to be enough specific for the galactose to avoid side reactions. In this study, a novel method for quantifying galactose was developed to serve as a newborn screening test for galactosemia using LDI-TOF MS with a TiO_2 nanowire chip. To quantitatively analyze galactose, the reduction potential of galactose was used to oxidize *o*-phenylene diamine (OPD) into 2, 3-diaminophenazine (DA), and both OPD and DA were quantitatively analyzed using LDI-TOF MS with a TiO_2 nanowire chip (Fig. 1a, g) (Fornera and Walde 2010; Hempen et al. 2005). To determine the feasibility of this method, the interference of glucose and proteins in the serum was determined and optical density measurements from EIA and MALDI-TOF MS analysis results were statistically analyzed. The simultaneous detection of galactose, amino acid, and thyroid hormone was also conducted, which demonstrated the possibility of employing a single test platform for newborn screening using MALDI-TOF MS based on a TiO_2 nanowire chip.

Methods/experimental

Materials

D-Galactose, D-glucose, fructose, maltose, sucrose, lactose, ethylenediaminetetraacetic acid (EDTA), OPD, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), sulfuric acid (H_2SO_4), acetonitrile, trifluoroacetic acid, phosphate citrate buffer tablet (PCB, pH 5.0, 50 mM), methanol, sodium hydroxide (NaOH), phenylalanine, 17α-hydroxyprogesterone (17α-OHP), human serum with no gene information were purchased from Sigma-Aldrich (St. Louis, MO, USA). Titanium plates ($30 \times 30 \times 1$ mm^3, ≥ 99.6% purity) were purchased from Goodfellow (Cambridge, England). The MSP 96 target plate for Micro Scout MALDI-TOF MS was purchased from Bruker Daltonics Co. (Billerica, MA, USA). Double-distilled deionized water (dw, 18 MΩ·cm) was prepared using the Milli-Q purification system (Millipore, Billerica, MA, USA).

Galactose–OPD assay

Galactose solutions were prepared in PCB at concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL. Galactose-spiked serum samples were also prepared at the same concentrations. OPD solution was prepared in PCB (1 mg/mL). Similarly, horseradish peroxidase solution was prepared in PCB at a concentration of 100 μg/mL. Sample solutions (70 μL) were dispensed, and OPD solution (140 μL) and horseradish peroxidase solution (5 μL) were added to each Eppendorf (EP) tube. Then, the solutions were incubated at 37 °C for up to 2 h. The OPD reaction was quenched by adding 0.5 M H_2SO_4 aqueous solution (50 μL). The optical density was measured at a wavelength of 430 nm every 10 min using a VersaMax ELISA Microplate reader (Sunnyvale, CA, USA).

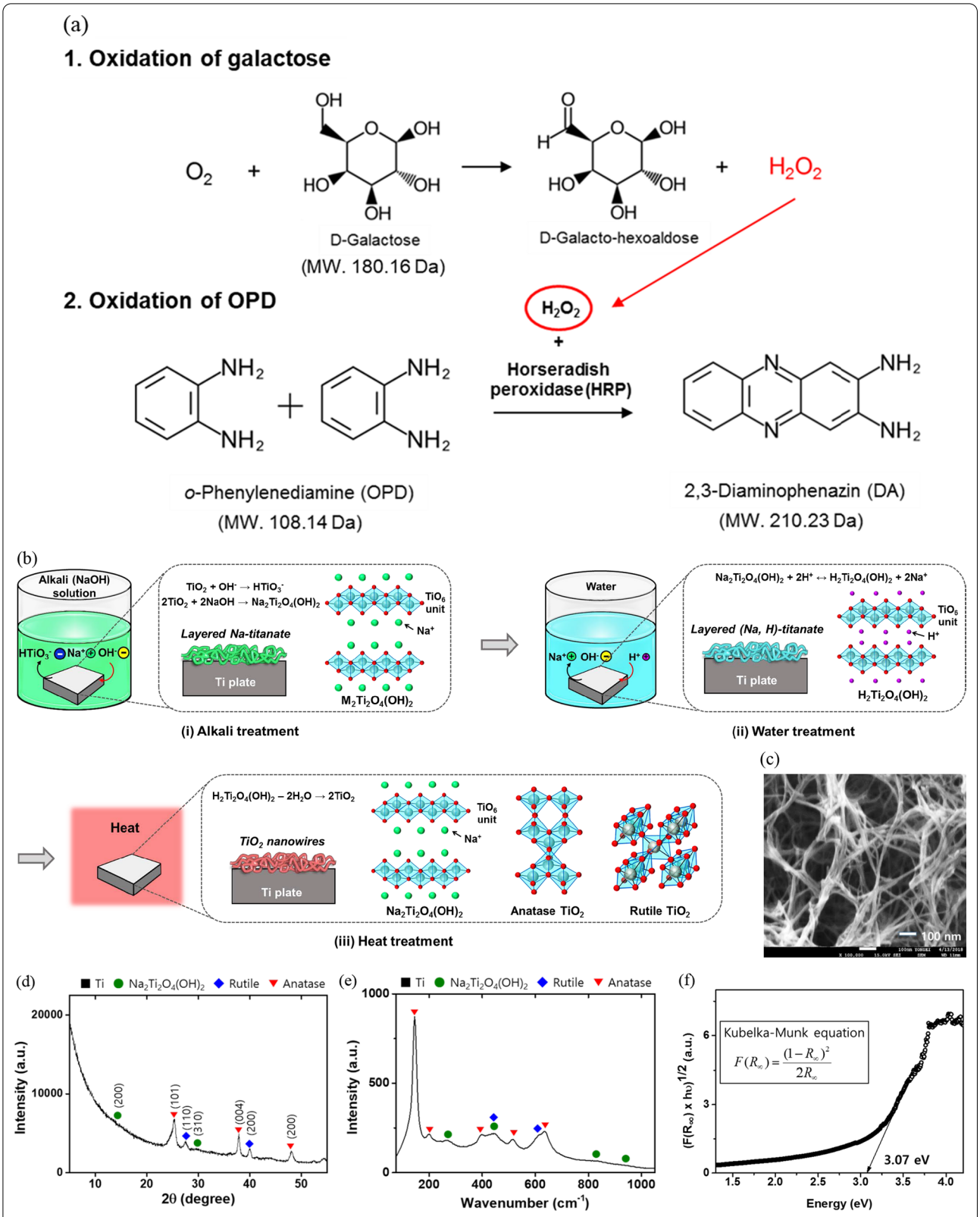

Fig. 1 Galactose analysis based on the reaction of *o*-phenylene diamine (OPD). **a** Diagram representing the reactions in the assay for galactose using OPD as a substrate. **b** Preparation of the TiO_2 nanowire chip. **c** FE-SEM image of the TiO_2 nanowire chip. **d** XRD pattern of the TiO_2 nanowire. **e** Raman spectrum of the TiO_2 nanowire. **f** Diffuse reflectance UV–vis spectra and Tauc plot of $(F(R_\infty) \times h\nu)^{1/2}$ versus $h\nu$ of the TiO_2 nanowire. **g** Mass spectra of galactose, OPD, and DA using MALDI-TOF MS based on conventional organic matrices (CHCA, DHB) and the TiO_2 nanowire chip

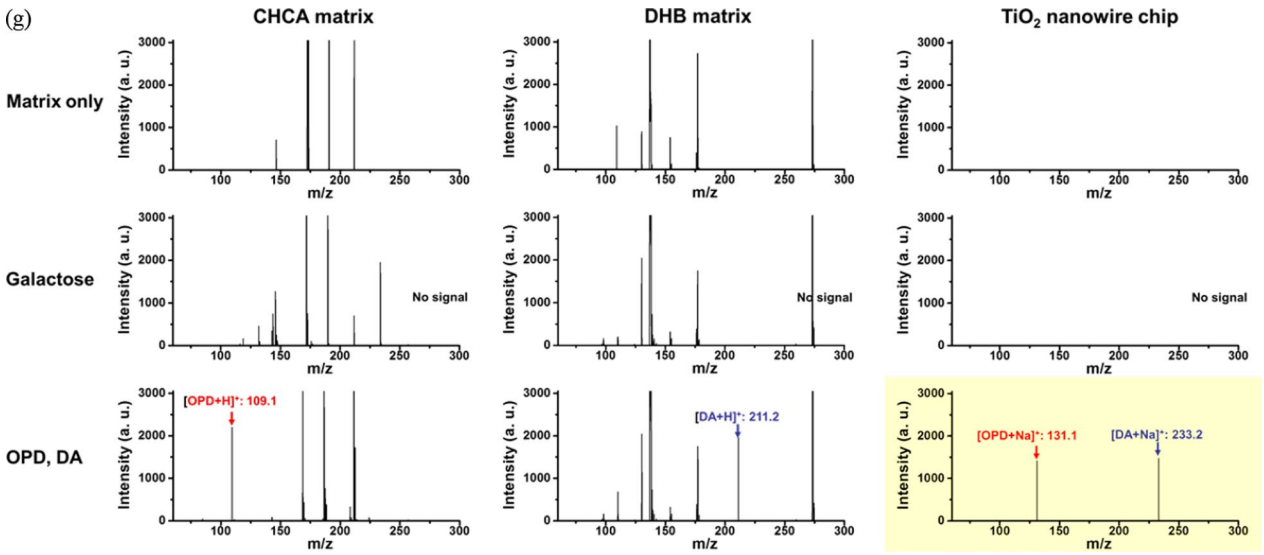

Fig. 1 continued

Fabrication of TiO_2 nanowire chip

LDI-TOF MS was carried out using TiO_2 nanowire plates on commercial steel target (MSP 96 target). Carbon tape was used to establish an electric contact between the Ti plate and stainless steel target.

TiO_2 nanowire chips were synthesized using the wet-corrosion process, which involves three sequential steps: (1) alkali treatment, (2) water treatment, and (3) heat treatment, as previously reported (Kim et al. 2014, 2016, 2020). The Ti plate was etched with a strong base, 10 M NaOH, for 24 h at room temperature with mild shaking. The Ti plate was rinsed 5 times with deionized water to eliminate the alkali solution and then dipped in deionized water for 48 h at room temperature with mild shaking. Finally, the Ti plate was thoroughly rinsed 3 times with deionized water and thermally annealed at 550 ℃ for 2 h in air.

The surface morphology of the TiO_2 nanowire chips was characterized using field-emission scanning electron microscopy (FE-SEM) with a microscope purchased from JEOL Co. (Tokyo, Japan). The crystal structure of the synthesized TiO_2 nanowires was characterized using high-resolution X-ray diffraction (HR-XRD; Rigaku, Japan) with Cu-Kα radiation in the Bragg–Brentano (θ/2θ) geometry. The surface of the Ti plate was scanned using a Raman spectrometer (HORIBA Jobin–Yvon, Longjumeau, France). The optical band gap energy was calculated using the diffuse reflectance value transformed according to the Kubelka–Munk function.

Detection of galactose–OPD assay products using MALDI-TOF MS with TiO_2 nanowire chip

Mass spectrometric detections were performed using the Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in reflective positive mode with first and second ion source voltages of 20 and 18 kV, respectively. The trigger frequency of a nitrogen laser was set to 60 Hz at a wavelength of 337 nm. The power of irradiated laser was adjusted to 40% of the maximum energy of 59.7 μJ with an offset of 35% and range of 37%. All mass spectra were obtained by integrating the data of 500 laser pulses for each sample spot. For LDI-TOF MS analysis, quenched galactose–OPD assay products (0.5 μL) were dispensed at five different spots on the surface of the TiO_2 nanowire chip. Thereafter, the sample-loaded chip was completely dried before analysis. Mean values of signal intensities from five different spots for the same concentration of galactose were used to calculate the signal. The measured intensities for each concentration were employed to plot graphs and were linearly approximated using the least-square methods. The limit of detection was threefold the noise level (Shrivastava and Gupta 2011). The reproducibility of LDI-TOF MS was verified using inter-spot and intra-spot analyses of galactose at the cutoff concentration. Inter-spot analysis was carried out at five different sample spots of galactose at a concentration of 110.0 μg/mL. The measurement was performed using the partial random walk function. Intra-spot analysis was also conducted in the same sample spot of galactose at a concentration of 110.0 μg/mL through five repeated measurements using the partial random walk function.

For the selectivity test under the interference of glucose, a mixture of galactose and glucose was analyzed using LDI-TOF MS. Galactose solutions were prepared at concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL, with a fixed glucose concentration of 541.0 μg/mL whereas glucose solutions were prepared at concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL, with a fixed galactose concentration of 33.4 μg/mL.

To demonstrate the feasibility of this newborn screening test, human serum was spiked with galactose at concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL. Galactose-spiked sera were diluted tenfold in cold methanol and extracted for 20 min. Serum was then centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant of the mixture was transferred to fresh EP tube and prepared for LDI-TOF MS analysis. Dried blood spot (DBS) samples were prepared using protein saver cards (Whatman # 903) from GE Healthcare Life Science Korea (Seoul, Korea). A protein saver card was punched into disks with a diameter of 6 mm. Punched disks were dipped into serum samples spiked with galactose at the known concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL. After 10 min of incubation, wet disks were transferred to fresh tubes and kept at room temperature to be air dried. Dried disks were used as DBSs in the following experiments. To extract galactose molecules from DBSs, dried disks were dipped into 87 μL of cold methanol and kept for 30 min at 4 °C. Thereafter, they were centrifuged at 12,000 rpm for 10 min at 4 °C for protein removal. Clear supernatants were then transferred to fresh EP tube for LDI-TOF MS analysis.

Mixtures of galactose, phenylalanine, and 17α-OHP were analyzed to evaluate the interference of other biomarkers of neonatal metabolic disorders. The concentrations of the analyte were changed whereas those of the other two biomarkers were fixed. For example, the concentration of phenylalanine was changed in the range around the cutoff value for diagnosing neonatal metabolic disorders, whereas the concentrations of galactose and 17-α-OHP were maintained at the same cutoff concentration.

Statistical analysis

MedCalc software version 18.6 for Windows was used for statistical analysis. LDI-TOF MS data are expressed as the mean ± standard deviation. To evaluate the correlations between the optical density from EIA using galactose oxidase and mass peak intensities from LDI-TOF MS based on the TiO_2 nanowire chip, the Bland–Altman test and Passing–Bablok regression were employed. For the EIA using galactose oxidase, galactose solutions were prepared in PCB at concentrations of 440.0, 330.0, 220.0, 192.5, 165.0, 137.5, 110.0, 82.5, 55.0, 27.5, and 0 μg/mL. Galactose oxidase solution was prepared in dw (3 mg/mL), horseradish peroxidase (HRP) solution was prepared in dw at a concentration of 80 μg/mL, and 3,3′,5,5′-tetramethylbenzidine (TMB) solution was prepared in aqueous solution at a concentration of 400 μg/mL. Sample solutions (100 μL) and galactose oxidase solution (50 μL) were added to a 96-well microplate and then incubated at 37 °C for up to 30 min. After H_2O_2 was expected to be produced because of galactose reduction, 100 μL of TMB and 10 μL of HRP were added to the 96-well microplate. TMB oxidation was quenched by adding 2 M H_2SO_4 aqueous solution (100 μL). Optical density was measured at a wavelength of 650 nm using VersaMax ELISA Microplate reader.

Results and discussion

Monosaccharides have been reported to be difficult to ionize using conventional matrix-assisted laser ionization method (Busse et al. 2006; Harvey 2015; Mechref et al. 2003; Pham et al. 2016). In particular, galactose was found to display strong reducing power among monosaccharides (Isbell et al. 1940; Lepilova et al. 2012; Srokol et al. 2004; Yang et al. 2008; Zhang et al. 2012; Zhou et al. 2020). In this study, the reducing power of galactose was used to oxidize OPD (molecular weight: 108.14 Da) to DA (molecular weight: 210.23 Da) (Fornera and Walde 2010; Hempen et al. 2005). The reducing power of galactose can be used to oxidize two molecules of OPD into one molecule of DA, and both OPD and DA were analyzed using LDI-TOF MS based on TiO_2 nanowire chips as shown in Fig. 1a.

For the quantitative analysis of OPD and DA, the TiO_2 nanowire chip was developed as a solid matrix for LDI-TOF MS. TiO_2 nanowire chips were synthesized using a wet-corrosion process comprising of three sequential steps: (1) alkali treatment, (2) water treatment, and (3) heat treatment (Fig. 1b). OPD and DA were both detectable under matrix-assisted laser ionization conditions and sodium-adducted mass peaks were detected using the TiO_2 nanowire chip. From the FE-SEM images, nanowire chips were observed to have a porous network structure with an average diameter of 20 nm (Fig. 1c).

Among the TiO_2 phases, the anatase and rutile phases are reported to be photo-catalytically active. The XRD patterns of TiO_2 nanowires showed mixed phases of anatase, rutile, and layered structures (Fig. 1d). To investigate the more specific crystal structure of TiO_2 nanowire, micro-Raman spectroscopic analysis was conducted. Based on the Raman spectra, titanium oxide synthesized with NaOH corrosion had layered titanates of the $Na_2Ti_2O_4(OH)_2$ type (Fig. 1e) (Kim et al. 2019, 2020). Photocatalytic activity and the large band gap (3.12 eV) of TiO_2 nanowire are reported to

effectively ionize the analyte using ultraviolet light during MALDI-TOF MS (Kim et al. 2018). Using the diffuse reflectance value, the optical band gap energy was determined to be 3.07 eV (Fig. 1f).

Galactose was impossible to detect directly using conventional organic matrix or a TiO_2 nanowire chip as shown in Fig. 1g. Compared to the conventional organic matrix, mass spectra using the TiO_2 nanowire chip showed separated mass peaks of OPD and DA without any noise in the low *m/z* range (Fig. 1g). For MALDI-TOF MS analysis, quenched galactose–OPD assay products were dropped on the surface of the TiO_2 nanowire chip. Using the TiO_2 nanowire as a solid matrix, we successfully ionized both OPD and DA in the sodium adduct form of $[M+Na]^+$ in the mass spectra profile with no interference of the matrix itself in the low *m/z* range (Fig. 1g), which corresponds with Raman analysis.

To demonstrate the feasibility of the method, the reproducibility of MALDI-TOF MS was verified using inter-spot and intra-spot analyses of galactose at the cutoff concentration. The reproducibility of inter-spot analysis of galactose was 3.72% for OPD and 6.40% for DA (Fig. 2a, Additional file 1: Figure S1a). The reproducibility of the intra-spot analysis of galactose was 3.49% for OPD and 7.43% for DA (Fig. 2b, Additional file 1: Figure S1b.

Galactose in PBS was quantitatively analyzed using MALDI-TOF MS, as shown in Fig. 3a; the mass intensities of OPD and DA were plotted against the concentration of galactose in buffer. MALDI-TOF MS spectra showed sodium-adducted mass peaks for OPD at an *m/z* ratio of $[OPD+Na]^+ = 131.1$ and DA at an *m/z* ratio of $[DA+Na]^+ = 233.2$. Furthermore, the intensities of the peaks changed quantitatively with the concentration of galactose in PCB, with good linearity ($r^2 = 0.986$ and 0.957) displayed in the range of 54.6–220.0 μg/mL. (Additional file 1: Figure S2a).

Detection of galactose levels in human blood and DBS samples provide useful information to assess liver function and diagnose galactosemia. Serum samples spiked with known concentrations of galactose were prepared, and methanol precipitation was carried out before MALDI-TOF MS analysis. Methanol extraction was conducted to precipitate most of the matrix proteins from the serum sample. Galactose in serum was also quantified using MALDI-TOF MS as shown in Fig. 3b and Additional file 1: Figure S2b. From the MALDI-TOF MS spectra, the intensities of the mass peaks of OPD and DA changed quantitatively with the concentration of galactose in serum. The intensities of the mass peaks of OPD and DA were linearly correlated with the concentration of galactose spiked in serum after OPD oxidation ($r^2 = 0.999$ and 0.950, respectively)

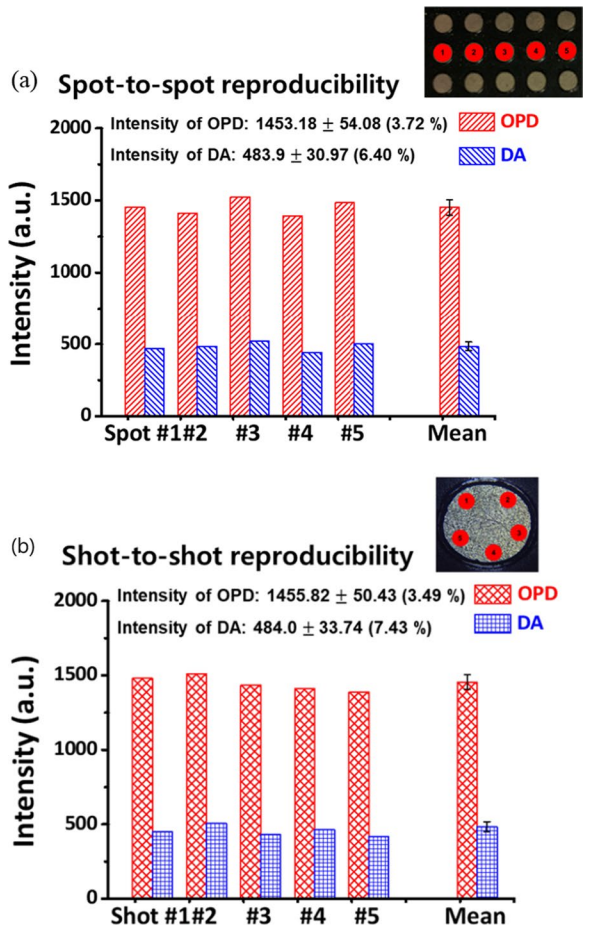

Fig. 2 Reproducibility of **a** inter-spot measurements for five independent sample spots ($n = 150$ shots) and **b** intra-spot measurements for five independent sample positions ($n = 150$ shots) using the TiO_2 nanowire chip

in the range of 57.2–220.0 μg/mL as shown in Figs. 3b and Additional file 1: Fig. S2c.

OPD oxidation was also applied for DBS and was performed using serum samples spiked with known concentrations of galactose dried on Whatman #903 filter paper. For the extraction of DBS, methanol precipitation was carried out and the oxidation of OPD was performed at galactose concentrations of 220.0, 165.0, 110.0, 55.0, and 0 μg/mL. Finally, galactose was quantified using MALDI-TOF MS as shown in Fig. 3c. MALDI-TOF MS spectra showed sodium-adducted mass peaks for OPD and DA, and the peak intensities changed quantitatively with the concentration of galactose in the serum dried on the filter paper. Peak intensities were linearly correlated with the concentration of galactose in the serum dried on the filter paper after OPD oxidation ($r^2 = 0.993$ and 0.985) from 52.5 to 220.0 μg/mL (Fig. 3c). Because the cutoff value for

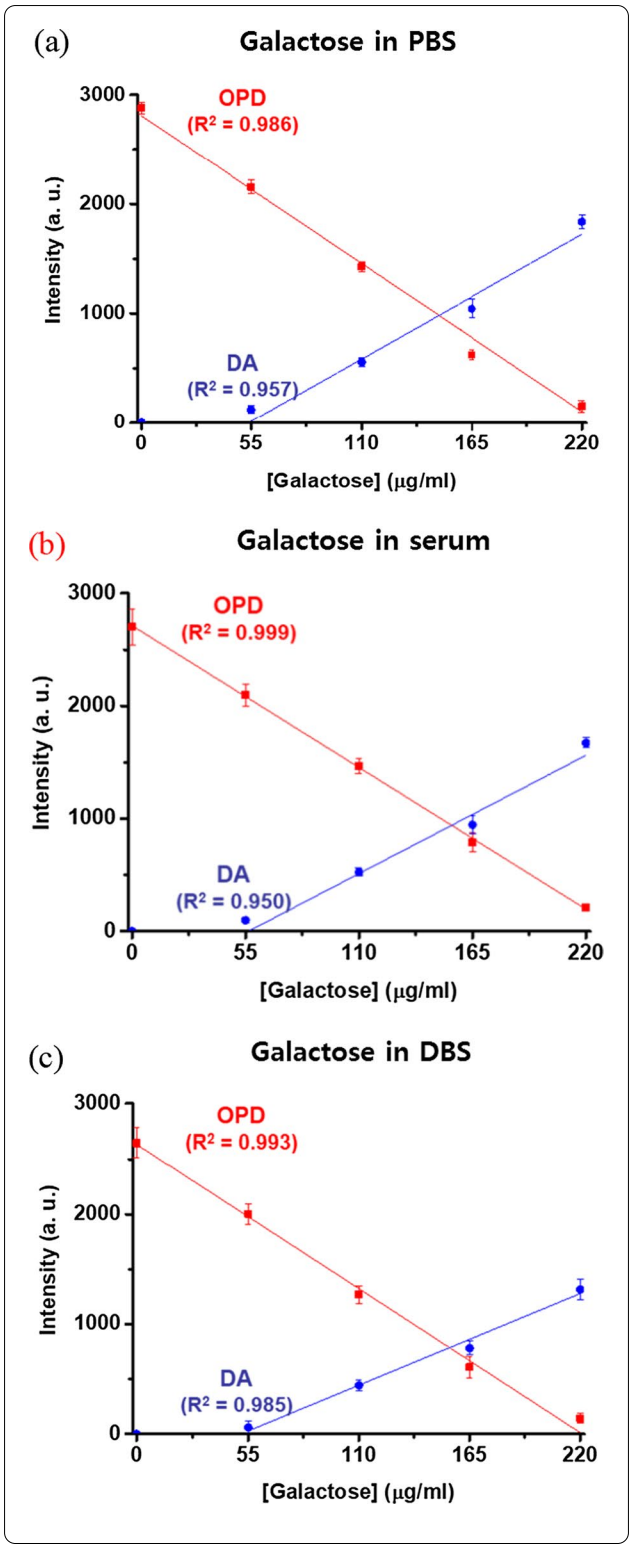

Fig. 3 Quantitative analysis of galactose using MALDI-TOF MS based on the TiO_2 nanowire chip. **a** Mass spectra of OPD assay products with different galactose concentrations in PCB. Correlation between the intensities of mass peaks of OPD and DA and the concentration of galactose. **b** Correlation between the intensities of mass peaks of OPD and DA and the concentration of galactose in serum after methanol extraction. **c** Correlation between the intensities of mass peaks of OPD and DA and the concentration of galactose in DBS after methanol extraction

medical diagnosis of galactosemia is 110.0 μg/mL, the results from serum samples as well as DBS showed that MALDI-TOF MS based on TiO_2 nanowire chip can be applied to diagnose galactosemia based on the oxidation of OPD with galactose.

As newborn screening for galactose is typically carried out using DBS from the whole blood of newborns, interference by other types of monosaccharides, particularly glucose, should be estimated before quantifying galactose. For the selectivity test under the interference of glucose, mixtures of galactose and glucose were subjected to OPD oxidation and the products were detected using MALDI-TOF MS (Additional file 1: Figure S3). The free galactose concentration in newborn plasma was reported to be 3.34 ± 2.23 mg/dL, whereas free glucose concentration was 54.1 ± 31.7 mg/dL (Araby et al. 2009; Dias and Gada 2014). Galactose solutions were prepared at different concentrations with a fixed glucose concentration (541.0 μg/mL), which is the average concentration of glucose in the newborn's plasma, and vice versa. The intensities of the mass peaks of OPD and DA changed quantitatively with the concentration of galactose at a fixed concentration of glucose in the mixture; however, an oxidation reaction by glucose at a fixed concentration of galactose was not significantly observed in the same concentration range as that of galactose (Fig. 4). Based on the comparison of these results, OPD oxidation was selectively carried out for galactose under fixed glucose conditions and could be analyzed using MALDI-TOF MS.

For comparison, the OD measurement of the EIA using galactose oxidase was derived using the galactose sample in PCB (Additional file 1: Figure S4). Correlation of the analysis results of MALDI-TOF MS based on the TiO_2 nanowire chip and the results of OD measurement from EIA were statistically analyzed using the Bland–Altman test and Passing–Bablok regression (Park et al. 2019).

Bland–Altman test revealed that a signal difference was distributed within the confidence level of 95% ($\pm 1.96\ 1\sigma$) (Fig. 5a). This result demonstrates that two methods are highly correlated and provide the same analysis result for the detection of galactose using EIA and MALDI-TOF MS. Passing–Bablok regression revealed that the analysis data from both methods were

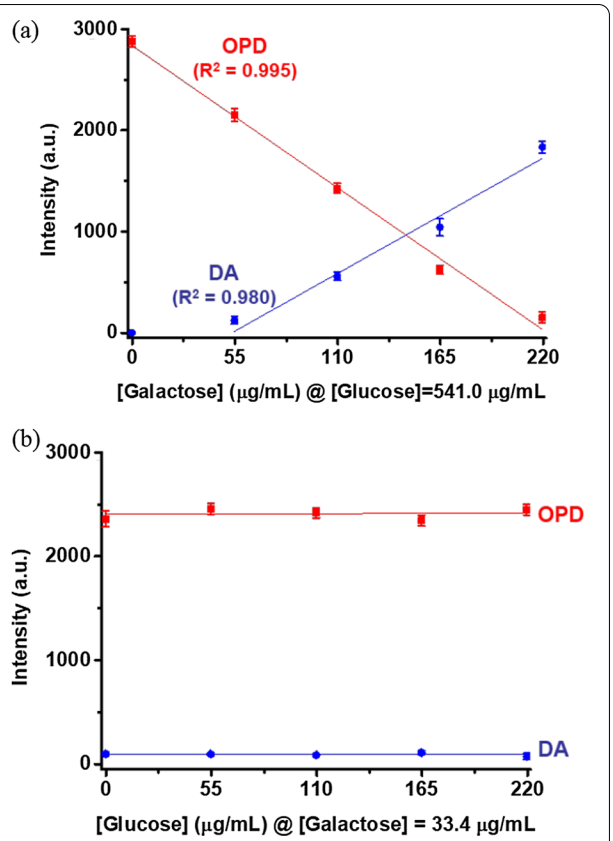

Fig. 4 Selectivity test of the OPD reaction with galactose concentration under the interference of glucose. **a** Mass spectra of OPD assay products with different concentrations of galactose and a fixed concentration of glucose (541.0 μg/mL). **b** Mass spectra of OPD reaction products with different concentrations of glucose and a fixed concentration of galactose (33.4 μg/mL)

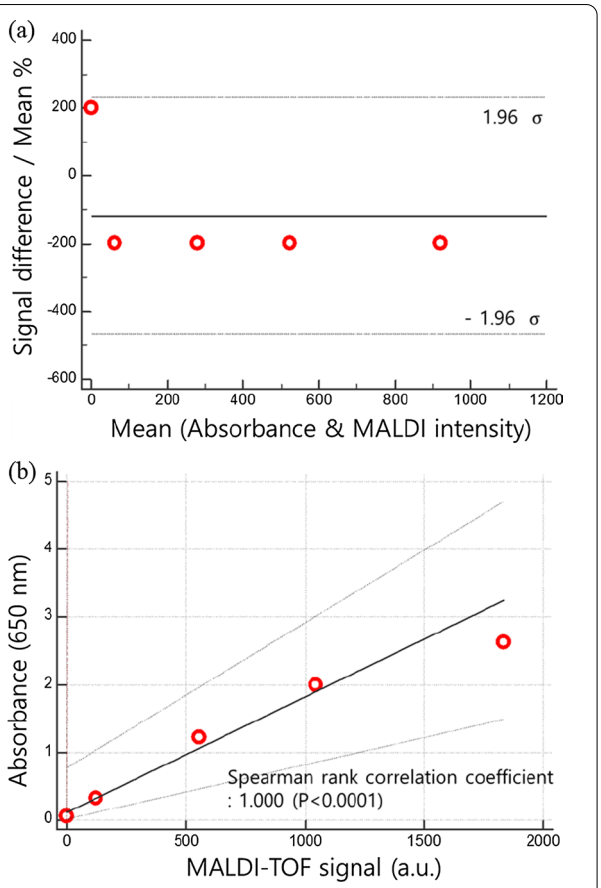

Fig. 5 Statistical analyses to estimate the consistency between MALDI-TOF MS based on TiO_2 nanowire chip and OD measurement from enzyme immunoassay using galactose oxidase in the **a** Bland–Altman test and **b** Passing–Bablok regression for the correlation analysis

distributed within a confidence level of 95%. Furthermore, the Spearman correlation coefficient (ρ) of 1.000 indicated that two different methods were statistically highly coincident (Fig. 5b).

Newborn screening tests have been used to diagnose diverse metabolic disorders in newborns by analyzing metabolites, such as amino acids, enzymes, and hormones. To evaluate the interference of other metabolites (biomarkers), mixtures of galactose, phenylalanine, and 17α-OHP were simultaneously detected using MALDI-TOF MS with a TiO_2 nanowire chip, demonstrating the feasibility of a single test platform for newborn screening. Phenylalanine was selected for the amino acid marker of phenylketonuria (PKU), and the cutoff was reported to be 4 mg/dL. Furthermore, 17-α-OHP was selected for the endocrine marker of congenital adrenal hyperplasia and the cutoff value was reported to be 15 ng/mL (Lee et al. 2008; Shoraka et al. 2020). The concentration of the analyte was changed whereas that of the other two biomarkers was fixed. For example, the concentration of phenylalanine was changed in the range around the cutoff value for diagnosing neonatal metabolic disorders, whereas the concentrations of galactose and 17-α-OHP were maintained the same at the cutoff concentration. Phenylalanine and 17-α-OHP were both quantitatively analyzed using MALDI-TOF MS with a TiO_2 nanowire as a solid matrix as shown in Fig. 6a. The intensities of OPD and DA changed quantitatively according to the galactose concentration, whereas those of the other phenylalanine and 17-α-OHP were the same (Fig. 6b). The results for phenylalanine and 17-α-OHP as the concentration-variable analytes showed the same trend (Fig. 6c, d). These results demonstrate that other biomarkers did not interfere in the analysis of galactose and the diagnosis of newborn metabolic disorders could be simultaneously be established using the multiplex test platform with MALDI-TOF MS based on a TiO_2 nanowire chip.

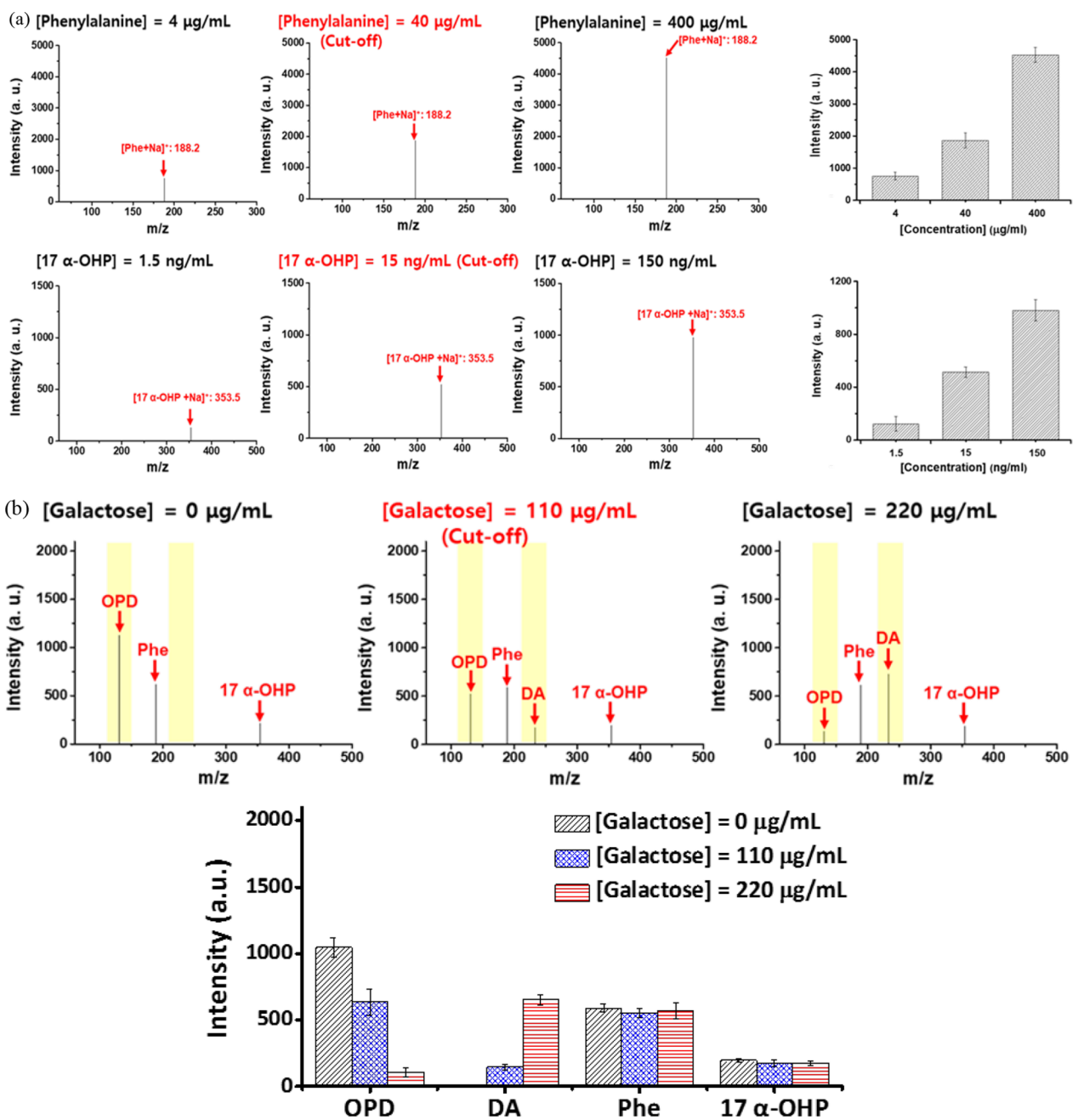

Fig. 6 Simultaneous analysis of galactose and other biomarkers (phenylalanine and 17α-OHP) for the newborn screening test. **a** Mass spectra of phenylalanine and 17α-OHP in PCB. **b** Mass spectra of OPD reaction products of mixtures with constant concentrations of phenylalanine and 17α-OHP; the concentration of galactose was changed in the range around the cutoff value. **c** Mass spectra of OPD reaction products of mixtures with constant concentrations of galactose and 17α-OHP; the concentration of phenylalanine was changed in the range around the cutoff value. **d** Mass spectra of OPD reaction products of mixtures with constant concentrations of phenylalanine and galactose; the concentration of 17α-OHP was changed in the range around the cutoff value

Conclusions

In this study, galactose in DBSs was quantified in a newborn screening test using MALDI-TOF MS based on TiO_2 nanowire chip to diagnose galactosemia. To quantify galactose, the reduction potential of galactose was used for the reaction of galactose with OPD to DA. When the TiO_2 nanowire chip was used as a solid matrix, both OPD and DA were ionized in the sodium adduct

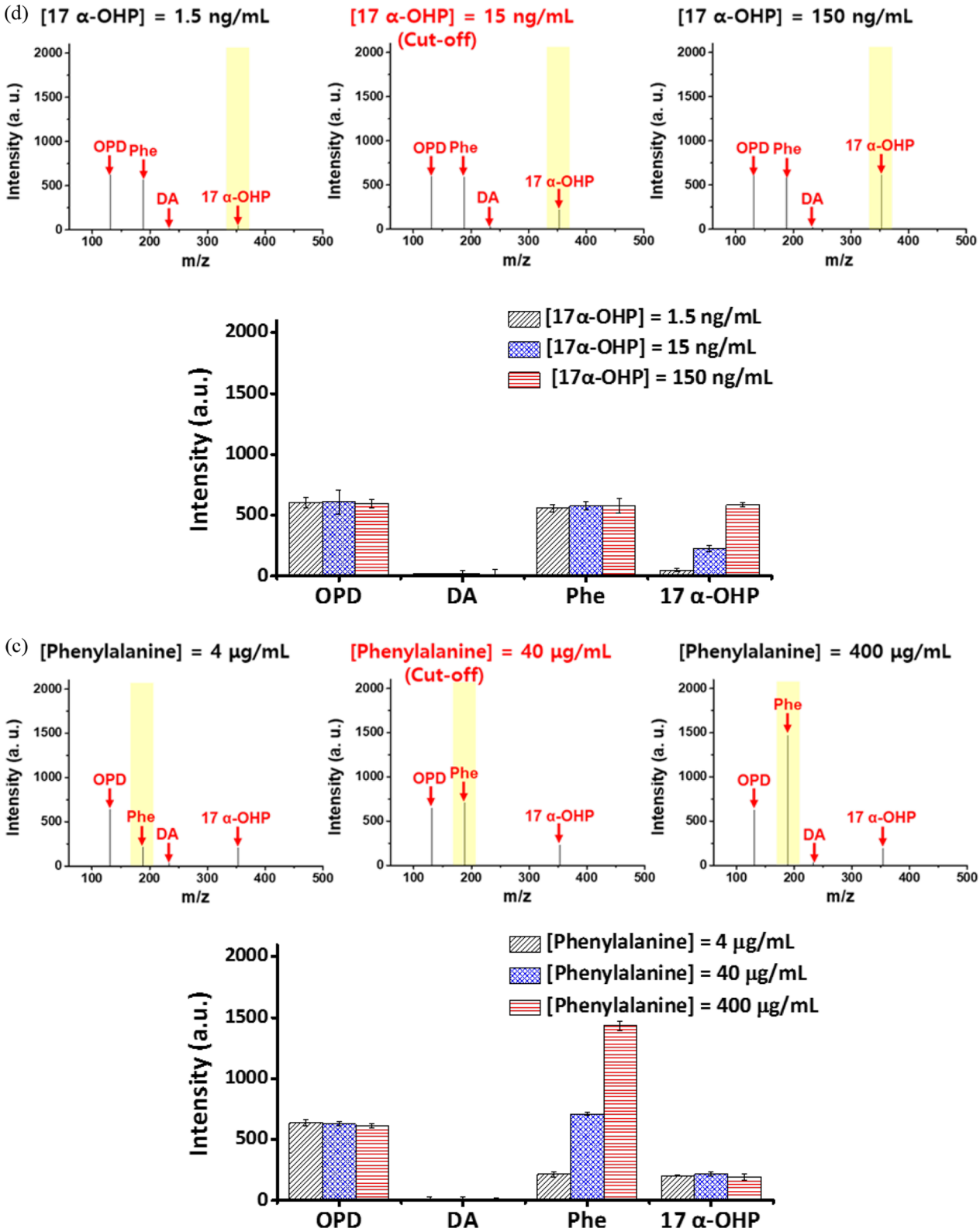

Fig. 6 continued

form of $[M+Na]^+$ in the mass spectra profile, with no matrix noise in the low m/z range. The reproducibility of the inter-spot and intra-spot analyses of galactose was within 10% for both OPD and DA. For serum and DBS samples, the OPD oxidation reaction was performed after methanol extraction to precipitate the matrix proteins in serum. Galactose in PBS, serum sample, and DBS was quantified using MALDI-TOF MS, and the intensities of the mass peaks of OPD and DA were linearly correlated in the galactose concentration range of 57.2–220.0 mg/mL ($r^2 = 0.999$ and 0.950, respectively) for serum samples and 52.5–220.0 mg/mL ($r^2 = 0.993$ and 0.985, respectively) for DBS. As the cutoff value for the medical diagnosis of galactosemia is 110.0 μg/mL, the results obtained using serum samples as well as DBS demonstrate that MALDI-TOF MS based on TiO_2 nanowire chip can be applied to diagnose galactosemia via the oxidation of OPD with galactose. To demonstrate the interference of glucose, a mixture of galactose and glucose was analyzed and an OPD reaction was selectively conducted for galactose. OD measurements from EIA and the MALDI-TOF MS results were statistically analyzed for the feasibility test. Finally, mixtures of galactose, phenylalanine, and 17α-OHP were simultaneously analyzed to determine the effect of other biomarkers of metabolic disorders in newborns, thereby displaying the potential of the multiplex test platform by MALDI-TOF MS based on a TiO_2 nanowire chip.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-021-00300-7.

Additional file 1. Figure S1. Reproducibility analysis using LDI-TOF Mass spectrometry based on TiO_2 nanowire chip. (**a**) Inter-spot measurements for five independent sample spots (n = 150 shots). (**b**) intra-spot measurements for five independent sample positions (n = 150 shots). **Figure S2**. Quantitative analysis of galactose using LDI-TOF MS based on the TiO_2 nanowire chip. (**a**) Mass spectra of OPD assay products at different galactose concentrations in PCB buffer. (**b**) Mass spectra of OPD assay products at different galactose concentrations in serum after methanol extraction. (**c**) Mass spectra of OPD assay products at different galactose concentrations in dried blood spot after methanol extraction. **Figure S3**. Selectivity analysis of the OPD assay for galactose measurement under the interference of glucose. (**a**) OPD assay at different concentrations of galactose and a fixed concentration of glucose (541.0 μg/mL). (**b**) OPD assay at different concentrations of glucose and a fixed concentration of galactose (33.4 μg/mL).

Acknowledgements
This work is supported by the National Research Foundation of Korea [grant numbers: NRF-2020R1A2B5B01002187, NRF-2020R1A5A101913111 and NRF-2021R1A2C209370611].

Authors' contributions
J.-Y.N. contributed to experiment, data curation, and writing-original draft. M.-J.K. contributed to experiment and data curation. M.K. contributed to experiment and data curation. J.-I.K. contributed to experiment and data curation. J.-M.P. contributed to experiment and data curation. T.G.Y. contributed to experiment and data curation. M.-J.K. contributed to supervision and review and editing. J.-C.P. contributed to supervision, funding acquisition, and writing-original draft, review and editing. All authors read and approved the final manuscript.

Funding
This work is supported by the National Research Foundation of Korea [Grant Numbers: NRF-2020R1A2B5B01002187, NRF-2020R1A5A101913111 and NRF-2021R1A2C209370611].

Availability of data and materials
Supporting information is supplied for reproducibility, mass spectra in different buffers, mass spectra of selectivity test and a standard curve of galactose using chromogenic reaction.

Declarations

Ethics approval and consent to participate
None.

Consent for publication
None.

Competing interests
No competing interests related to this paper.

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Received: 23 August 2021 Accepted: 6 October 2021
Published online: 02 November 2021

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