Original Research

Matrix effects break the LC behavior rule for analytes in LC-MS/MS analysis of biological samples

Nianbai Fang[1,2], Shanggong Yu[2], Martin JJ Ronis[1,2,3] and Thomas M Badger[1,2,4]

[1]Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA; [2]Arkansas Children's Nutrition Center, Little Rock, AR 72202, USA; [3]Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA; [4]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Corresponding author: Thomas M Badger. Email: BadgerThomasM@uams.edu

Abstract

High-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are generally accepted as the preferred techniques for detecting and quantitating analytes of interest in biological matrices on the basis of the rule that one chemical compound yields one LC-peak with reliable retention time (Rt.). However, in the current study, we have found that under the same LC-MS conditions, the Rt. and shape of LC-peaks of bile acids in urine samples from animals fed dissimilar diets differed significantly among each other. To verify this matrix effect, 17 authentic bile acid standards were dissolved in pure methanol or in methanol containing extracts of urine from pigs consuming either breast milk or infant formula and analyzed by LC-MS/MS. The matrix components in urine from piglets fed formula significantly reduced the LC-peak Rt. and areas of bile acids. This is the first characterization of this matrix effect on Rt. in the literature. Moreover, the matrix effect resulted in an unexpected LC behavior: one single compound yielded two LC-peaks, which broke the rule of one LC-peak for one compound. The three bile acid standards which exhibited this unconventional LC behavior were chenodeoxycholic acid, deoxycholic acid, and glycocholic acid. One possible explanation for this effect is that some matrix components may have loosely bonded to analytes, which changed the time analytes were retained on a chromatography column and interfered with the ionization of analytes in the MS ion source to alter the peak area. This study indicates that a comprehensive understanding of matrix effects is needed towards improving the use of HPLC and LC-MS/MS techniques for qualitative and quantitative analyses of analytes in pharmacokinetics, proteomics/metabolomics, drug development, and sports drug testing, especially when LC-MS/MS data are analyzed by automation software where identification of an analyte is based on its exact molecular weight and Rt.

Keywords: Liquid chromatography-tandem mass spectrometry, matrix effect, LC behavior, bile acid, piglet urine

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Introduction

Liquid chromatography–mass spectrometry (LC-MS) is an analytical technique that combines the physical separation capabilities of high-performance liquid chromatography (HPLC or LC) with the mass analysis capabilities of mass spectrometry. The technique is oriented towards the detection and quantification of analytes of interest in complex matrices, and is widely used in studies of pharmacokinetics, proteomics/metabolomics, drug development, and sports drug testing because of its very high sensitivity and selectivity. Although the common perception is that utilization of LC-MS practically guarantees specificity and reliability for the detection and quantitation of chemicals, the LC-MS methodology does encounter problems caused by matrix effects.[1] A matrix effect is defined as the overall consequence of all components in the sample other than the analyte of interest.[2–4] Matrix components include endogenous and exogenous factors. Some matrix effects are well known, but many remain undetermined.[5–11] Matrix effects are extremely diverse and are dependent on the analyte, LC-MS method, and ion source.[4] In LC-MS analysis, the matrix components can be co-eluted with the analyte of interest and interfere with the ionization process of the analyte in the mass spectrometer. This matrix effect can cause ionization suppression or enhancement, which adversely affects the results of LC-MS analysis, and leads to erroneous reporting of analyte quantitation.[4] Since commonly encountered matrix effects are ionization enhancements or suppressions, studies on matrix effect mechanisms have focused on the

ionization of analytes, and have suggested that matrix effects arise at the interface (ion source) between the LC system and the MS system.[4] The ESI source has been reported to be more vulnerable to matrix effects when compared to either APCI or APPI because of the acquisition of charge in the solution phase and transitioning to the gas phase in ESI source.[12–16]

Our preliminary study on bile acids in urine revealed that contrary to common perceptions, matrix effects are not limited to only ionization enhancement or suppression that adversely affects the quantitation of analytes. Specifically, we found that matrix effects can also significantly change the retention time (Rt.) of LC-peaks which can lead to erroneous conclusions in the detection of analytes of interest when the Rt. of the LC-peaks of their corresponding standards are used as a constant property of the analyte under the same LC conditions. The purpose of the present work is to verify and characterize this aspect of the matrix effect. Urine from four groups of young pigs was used as sources of matrix effects, and 17 authentic bile acid standards were used as analytes to evaluate the degree and specificity of the matrix effects. The bile acids currently under investigation in our laboratories are the major metabolites of cholesterol and play an important physiological role in the elimination of cholesterol from the body, and the absorption of dietary lipids and fat-soluble vitamins.

Materials and methods

Materials

Seventeen authentic standards of bile acids were used in the study. Nine of the standards were purchased from Steraloids, Inc. (Newport, RI, USA): α-muricholic acid (α-MCA), β-muricholic acid (β-MCA), γ-muricholic acid (γ-MCA), sodium glycochenodeoxycholate (GCDCA sodium salt), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), and taurolithocholic acid (TLCA). Eight of the standards were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), hyodecholic acid (HDCA), lithocholic acid (LCA), glycocholic acid (GCA), and glycoursodeoxycholic acid (GUDCA).

Methanol (Spectranalyzed®) and acetonitrile (Chromasolv®) were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Water was obtained from Milli-Q Integral Water Purification System (EMD Millipore Corporation, Billerica, MA, USA).

Animal experiments

Pig experiments were performed as previously described.[17,18] Briefly, White × Dutch Landrace × Duroc sows were artificially inseminated, and following birth were allowed to suckle for 48 h before being randomly assigned to either breast-fed or formula-fed groups of approximately equal mean weight. Male and female breast-fed piglets (Sow) remained with sows for the duration of the experiment and allowed to breast feed *ad libitum*. Other male and female piglets were fed cow's milk-based formula (Milk) (Similac Advance powder; Ross Products, Abbott Laboratories, Columbus, OH, USA). Formula-fed piglets were trained to drink from small bowls on a fixed schedule as described previously until sacrifice on postnatal day (PND) 21.[19] Formula diets were modified to meet the energy and nutrient recommendations of the National Research Council (NRC) for growing pigs and the formula diet composition has been previously published.[17] All animals were housed in the animal facilities of the Arkansas Children's Hospital Research Institute, an Association for the Assessment and Accreditation of Laboratory Animal Care-approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences (Little Rock, AR, USA). Pigs were killed by exsanguination after anesthetization with isoflurane at 0800-1000 h, 6–8 h after the final feeding period and urine samples were obtained from the bladder at sacrifice.

Urine specimens

Pooled urine samples (urine from $n = 5$ piglets/pool) were used as sources of matrix components and designated as follows: cow's milk formula-fed males (P1 urine), cow's milk formula-fed females (P2 urine), breast-fed males (P3 urine), or breast-fed females (P4 urine).

LC-MS/MS sample preparation

Each urine sample (1.0 mL) was put in a 1.5 mL microcentrifuge tube and frozen at −80°C followed by lyophilization. The powdered urine in the tube was added with 1 mL of methanol and vortexed vigorously for 2 min followed by sonication in ice water for 5 min. The mixture was centrifuged at RCF 153,393 *g* for 5 min, and the supernate was filtrated through a 17-mm 0.2 μm filter (National Scientific, TN, USA). The extraction process was repeated one more time with 1.0 mL of 100% methanol, and the extracts were combined. The four methanol extract solutions prepared from samples of P1, P2, P3, and P4 urine were labelled P1, P2, P3, and P4, respectively. The 17 bile acid standards (Figure 1) were divided into three groups based on their chemical structures: the unconjugate group consisting of the nine unconjugates; the glycine conjugate group consisting of the five glycine conjugates; and the taurine conjugate group consisting of the three taurine conjugates. For qualitative and quantitative determination of matrix effects, each group was dissolved in P1, P2, P3, P4, and pure methanol, respectively, at concentrations of 30, 100, 300, 1000, or 3000 pmol/mL. The urine components in all experimental samples were adjusted to the same concentration equivalent to 0.5 mL urine/mL with methanol.

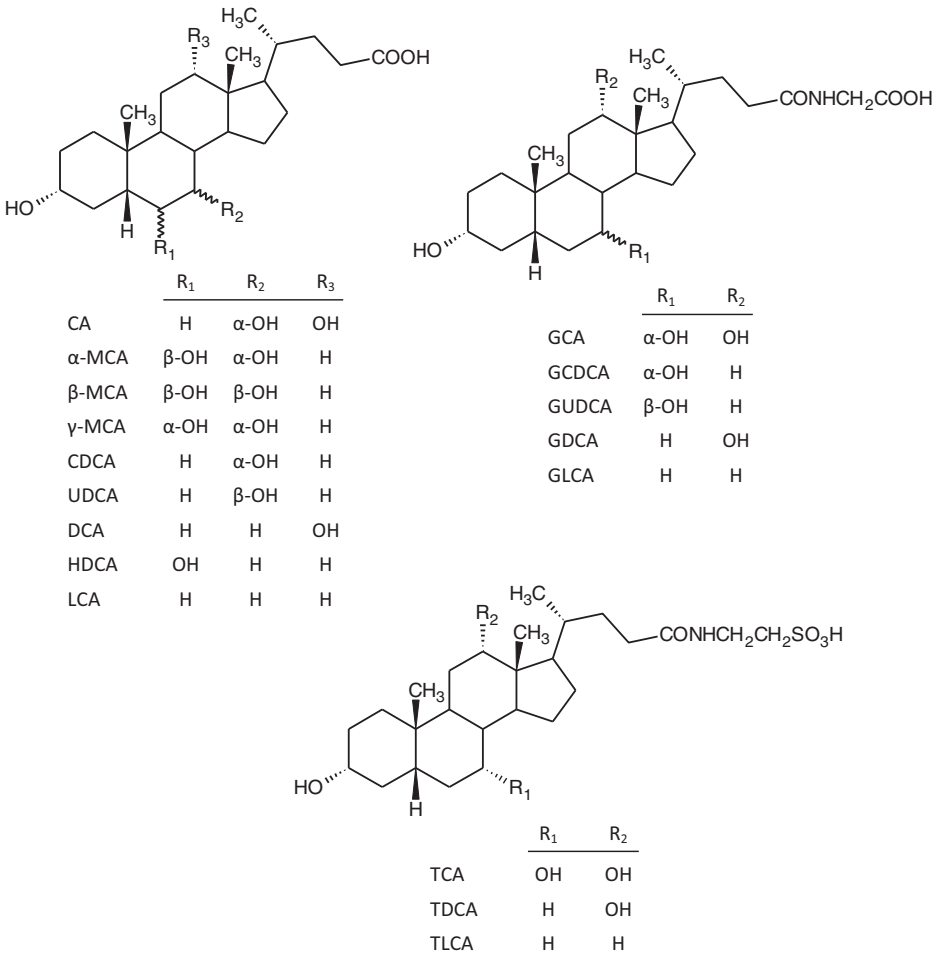

Figure 1 Chemical structures of bile acid standards used in this study

LC-MS/MS analysis

A 10 μL aliquot of the sample (equivalent to 5 μL of urine) was directly analyzed by LC-MS/MS. LC-MS/MS was performed using a 4000 Q TRAP system (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent 1100 series liquid chromatograph (Agilent Technologies, Wilmington, DE, USA). The 4000 Q TRAP system includes a hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer equipped with an ESI probe and Analyst® software. A 150 mm × 2 mm i.d. Synergi 4 μ Fusion-RP 80 A column (Phenomenex, Torrance, CA, USA) was used with LC solvent at a flow rate of 0.5 mL/min. The LC gradient was 5% acetonitrile/methanol (solvent B) in water (solvent A) as follows: 45–50% in 10 min; 50–70% from 10 to 12 min; 70–85% from 12 to 21 min; 85–100% from 21 to 22 min; held at 100% from 22 to 25 min and finally back to 45% at 29 min, with a min as column re-equilibration in sequence analysis. The same bile acid group in different samples was analyzed by LC-MS/MS under the same MS conditions. Bile acids in the eluate were monitored by MRM (Multiple Reaction Monitoring) scan with a negative ion mode (Table 1). All parameters other than CE (Table 1) for ESI-MS analysis of HPLC peaks were held constant: DP, −125.0; EP, −10; and CXP, −10.0. Parameters for source/gas were: CUR, 10.0; CAD, MEDIUM; IS, −4500.0; TEM, 400.0; GS1, 18.0; and interface heater is On.

Table 1 Multiple reaction monitoring scan with a negative ion mode

	Q1 Mass (Da)	Q3 Mass (Da)	CE (eV)	ID
Condition for unconjugate group:				
1	407.2	343.2	−40	CA
2	407.2	389.4	−40	α-MCA, β-MCA, γ-MCA
3	391.3	373.2	−40	CDCA, UDCA, HDCA
4	391.3	345.5	−40	DCA
5	375.2	357.2	−40	LCA
Condition for glycine conjugate group:				
1	464.3	74.0	−50	GCA
2	448.3	74.0	−50	GCDCA, GUDCA, GDCA
3	432.3	74.0	−50	GLCA
Condition for taurine conjugate group:				
1	514.3	124.0	−55	TCA
2	498.3	124.0	−55	TDCA
3	482.3	124.0	−55	TLCA

Statistical analysis

Data are expressed as means ± SD for at least three replicate determinations. Statistical differences were analyzed by one-way or two-way ANOVA followed by *post hoc* analysis performed with the statistical software from SigmaPlot

(Systat Software, San Jose, CA, USA). One-way ANOVA was used in comparison between data from standards without and with matrix interferer (std. in methanol vs. std. in P1, P2, P3, or P4). For the differential matrix effects of gender and diet, two-way ANOVA was used. A P value of less than 0.001 was considered as statistically significant.

Results

Strategy for evaluation of matrix effects from urines

The methanol extract solutions prepared from four urine pools (P1, P2, P3, and P4) were used as sources of matrix components with the aim of determining the influence of two factors, diet and gender, in the matrix effects. Three groups of bile acid standards (Figure 1) were used as analytes to evaluate the degree and specificity of the matrix effects. For qualitative and quantitative determination of matrix effects, each group was dissolved in P1, P2, P3, P4, and pure methanol, respectively. The concentrations of urine components in all experimental samples were equivalent to 0.5 mL urine/mL, and the standards were dissolved in methanol containing urine extract or pure methanol in different concentration levels. In order to facilitate comparison, the same LC conditions were used for all samples and the same group of standards in different samples was analyzed under the same MS conditions. Since matrix effects can arise from mobile phase additives such as formic acid or ammonium hydroxide,[4] H_2O, methanol, and acetonitrile without any additives were used in the solvent system of the LC-MS/MS analysis. The three groups of bile acids in pure methanol were analyzed by LC/MS/MS in MRM scan mode to obtain good peak shape and separation as well as proper Rt. between 14.27 min and 22.69 min (Figures 2 and 3). The matrix effects of urine on Rt. of the LC-peaks of bile acids were quantitated by direct comparison between the corresponding standards in methanol with and without the extract of pig urine (Figures 2–5).

Matrix effects on LC behavior

The Rt. and shape of the LC peak of an analyte, which for convenience we will refer to as the analyte's ''LC behavior,'' are properties of chemicals used to detect an analyte of interest by comparison with the LC behavior of the analyte's standard in HPLC and LC-MS/MS analyses. There are no published data about matrix effects on LC behavior. For the nine unconjugated bile acids, matrix components in urine from cow's milk formula-fed piglets (P1 and P2) significantly reduced the Rt. of the LC- peaks of all nine bile acids ($P < 0.001$). The degree of potency of the matrix effects on the Rt. of the LC-peaks of these nine unconjugated bile acids was only minimally influenced by the number, positions, and orientations of hydroxyls in their chemical structures and their hydrophilic–hydrophobic index (HHI).[20–22] In contrast to P1 and P2, the urine from breast-fed piglets (P3 and P4) did not show significant matrix effect on the Rt. of the LC-peaks of the nine bile acids (Figure 4).

The matrix effects of P1 and P2 on the Rt. of the LC-peaks of the conjugated bile acids were similar to their effects on that of the unconjugated bile acids in that the matrix components in P1 and P2 significantly reduced the time that the all conjugated bile acids were retained on the chromatography column ($P < 0.001$). However, the potency of matrix effect of P1 and P2 on the conjugates differed. The degree of potency of the matrix effects of P1 and P2 on the Rt. of the LC-peaks of the conjugated bile acids increased as the hydrophobicity index (HIx) of the bile acid decreased with the exception of GLCA. In other words, the Rt. of the LC-peaks of the conjugated bile acids with smaller HIx[23] was affected to a larger degree by the matrix effects of P1 and P2, i.e. GUDCA $(-0.43) >$ GCA $(0.07) >$ GLCA $(1.05) >$ GDCA $(0.65) \approx$ GCDCA (0.51) for the glycine conjugates, and TCA $(0.00) >$ TDCA $(0.59) \approx$ TLCA (1.00) for the taurine conjugates (Figure 5), where the inequality signs indicate the degree of potency and the numbers in parentheses are HIx. It has been previously reported that the LC-peak areas of the more highly polar analytes tend to be affected to a greater degree by matrix effects than that of the less polar molecules.[4] Taurine conjugates of the bile acids are known to be more hydrophilic than the glycine ones.[20–22] However, we observed in the present study that the degree of matrix effects on the Rt. of the LC-peaks of the taurine conjugates was significantly lesser than that of the corresponding glycine conjugate (Figure 5A and E). Unlike the unconjugated bile acids which were not affected by P3 and P4, there was a significant diversity of matrix effects of P3 and P4 on the LC behavior of the conjugated bile acids. While the matrix components of P3 and P4 significantly decreased the Rt. of the LC-peaks of GUDCA and TCA, there was no matrix effect on the Rt. of the LC-peaks of GDCA, GCDCA, and GLCA. And although the matrix components of P3 and P4 only slightly decreased the Rt. of TDCA and TLCA, statistical analysis by one-way ANOVA indicated that these decreases of Rt. were still significant ($P < 0.001$) (Figure 5A and E).

A matrix effect of particular interest was one that resulted in a LC behavior whereby a single compound yielded two LC-peaks, while it is generally expected that one compound will yield one LC-peak. The matrix effects of P1 and P2 on DCA and CDCA yielded two peaks for each compound with significant decreases in the Rt. of the peaks (Figure 4A and B). The matrix effects of P3 and P4 on GCA yielded two peaks where the Rt. of one of the peaks was not affected while the Rt. of the other peak was decreased (Figure 5A).

When gender and diet were used as factors for statistical comparison of the matrix effects of P1, P2, P3, and P4 on Rt. of LC-peaks by two-way ANOVA, it was determined that there was not a statistically significant difference between genders ($p = 0.377$), whereas there was a statistically significant difference between diets ($P < 0.001$). The matrix components in P1 and P2 (extracts prepared using urine from cow's milk formula-fed piglets) significantly changed the LC behavior of all bile acids ($P < 0.001$). On the other hand, we found that the extent of the matrix effects of P3 and P4 (extracts prepared using urine from breast-fed piglets) differed significantly among the bile acids, and the potency of the overall matrix effect was much smaller than that of P1 and P2.

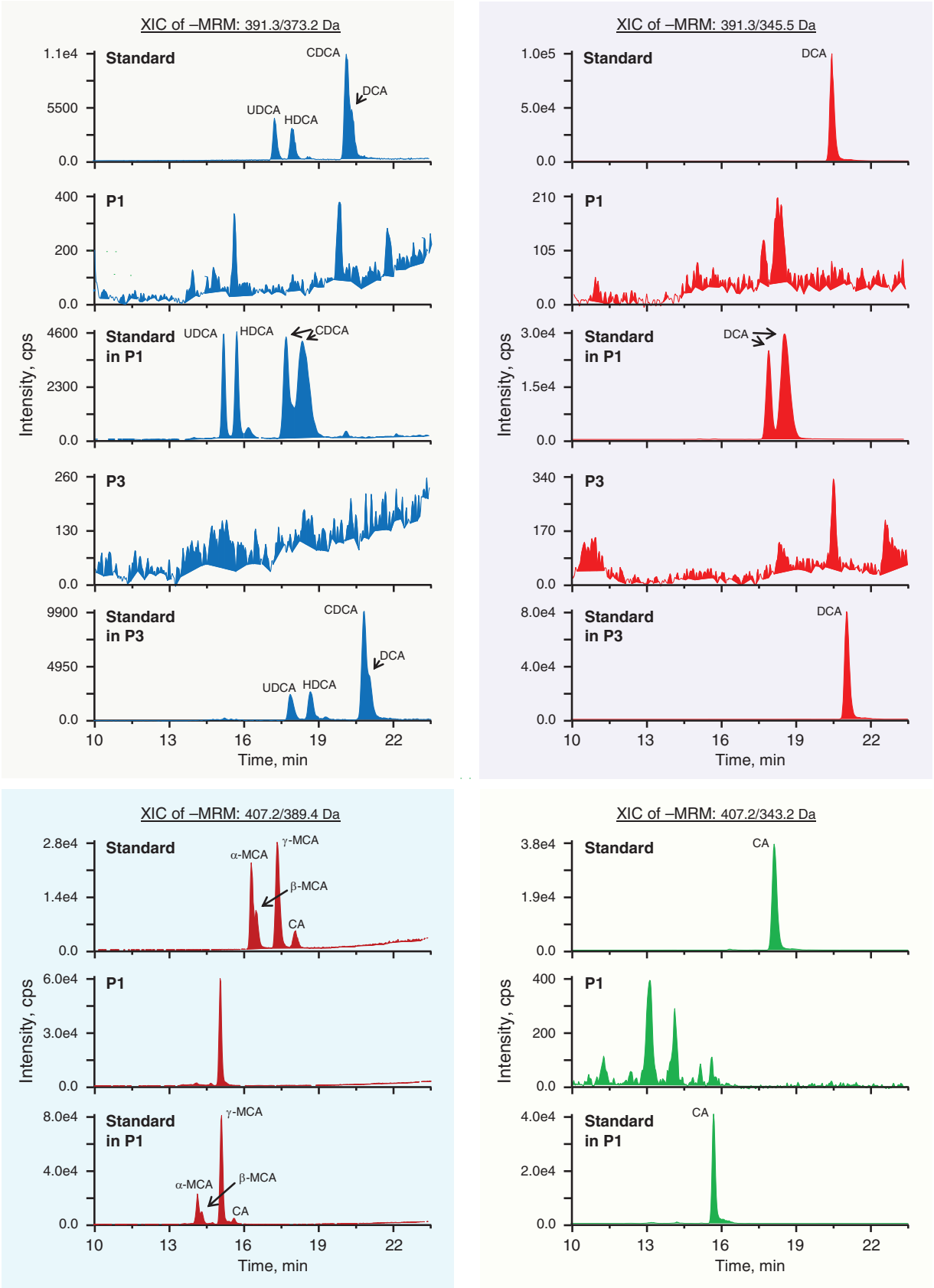

Figure 2 Multiple reaction monitoring (MRM) scan chromatograms of unconjugated bile acid standards. Standard, chromatogram of standards in pure methanol; P1, chromatogram of P1 without standards (P1 is a methanol extract of urine from cow's milk formula-fed male piglets); Standard in P1, chromatogram of standards in P1; P3, chromatogram of P3 without standards (P3 is a methanol extract of urine from breast-fed male piglets); and Standard in P3, chromatogram of standards in P3. The XIC of –MRM 391.3/373.2, 391.3/345.5, 407.2/389.4, and 407.2/343.2 Da are shown in blue, red, dark red, and green, respectively. (A color version of this figure is available in the online journal.)

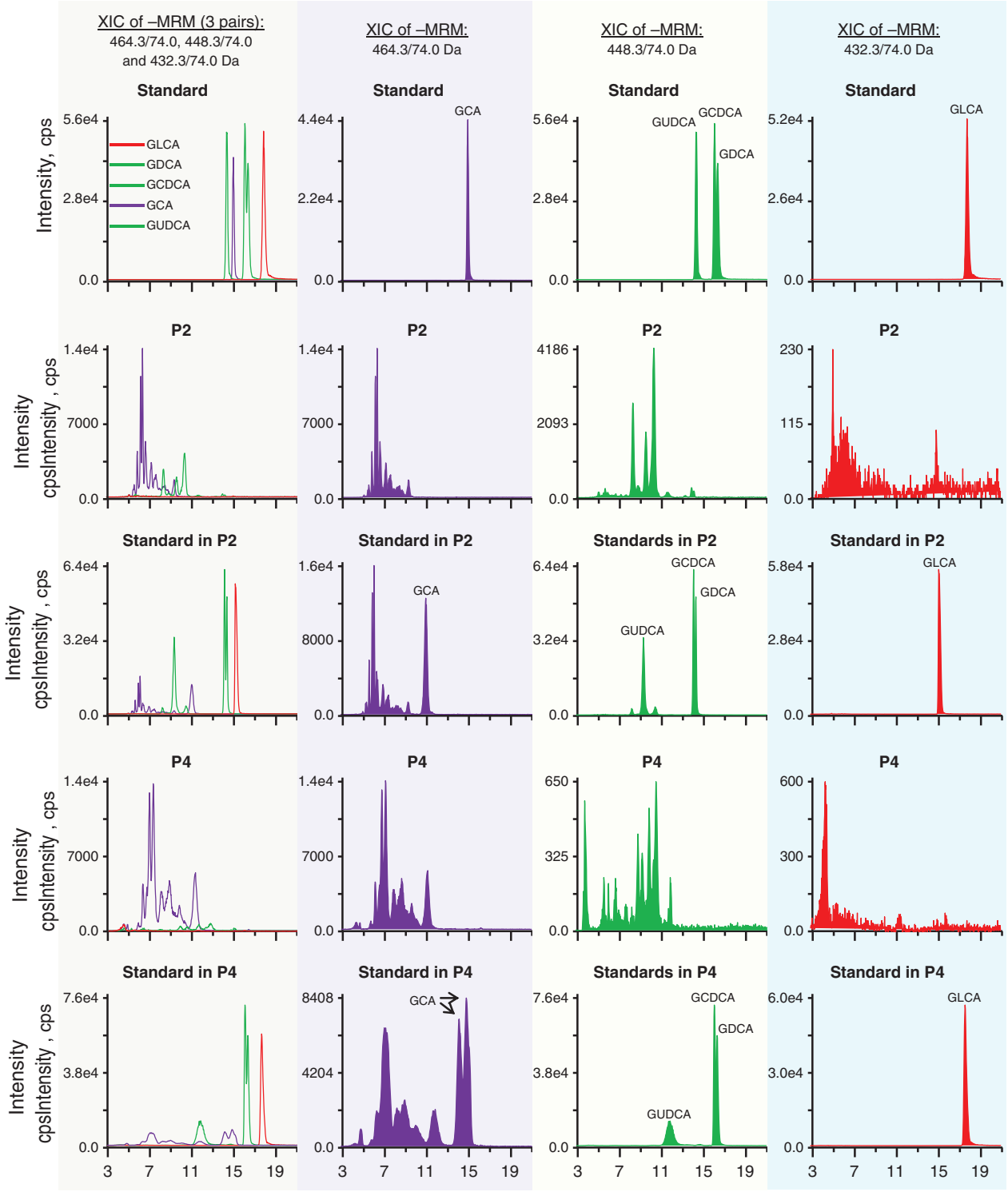

Figure 3 Multiple reaction monitoring (MRM) scan chromatograms of glycine-conjugated bile acid standards. Standard, chromatogram of standards in pure methanol; P2, chromatogram of P2 without standards (P2 is a methanol extract of urine from cow's milk formula-fed female piglets); Standard in P2, chromatogram of standards in P2; P4, chromatogram of P4 without standards (P4 is a methanol extract of urine from breast-fed female piglets); and Standard in P4, chromatogram of standards in P4. (A color version of this figure is available in the online journal.)

Matrix effects on MS ionization

There have been numerous studies of matrix effects on ionization of analytes and the adverse effects on quantitative results caused by ion enhancement or suppression.[4] We also observed this phenomenon of the matrix effects on the LC-peak areas of bile acids in the present study. The matrix components in P1, P2, P3, and P4 decreased the LC-peak areas of five of the unconjugated bile acids. For the remaining four unconjugates, matrix components increased the peak areas of α-MCA and γ-MCA, and did

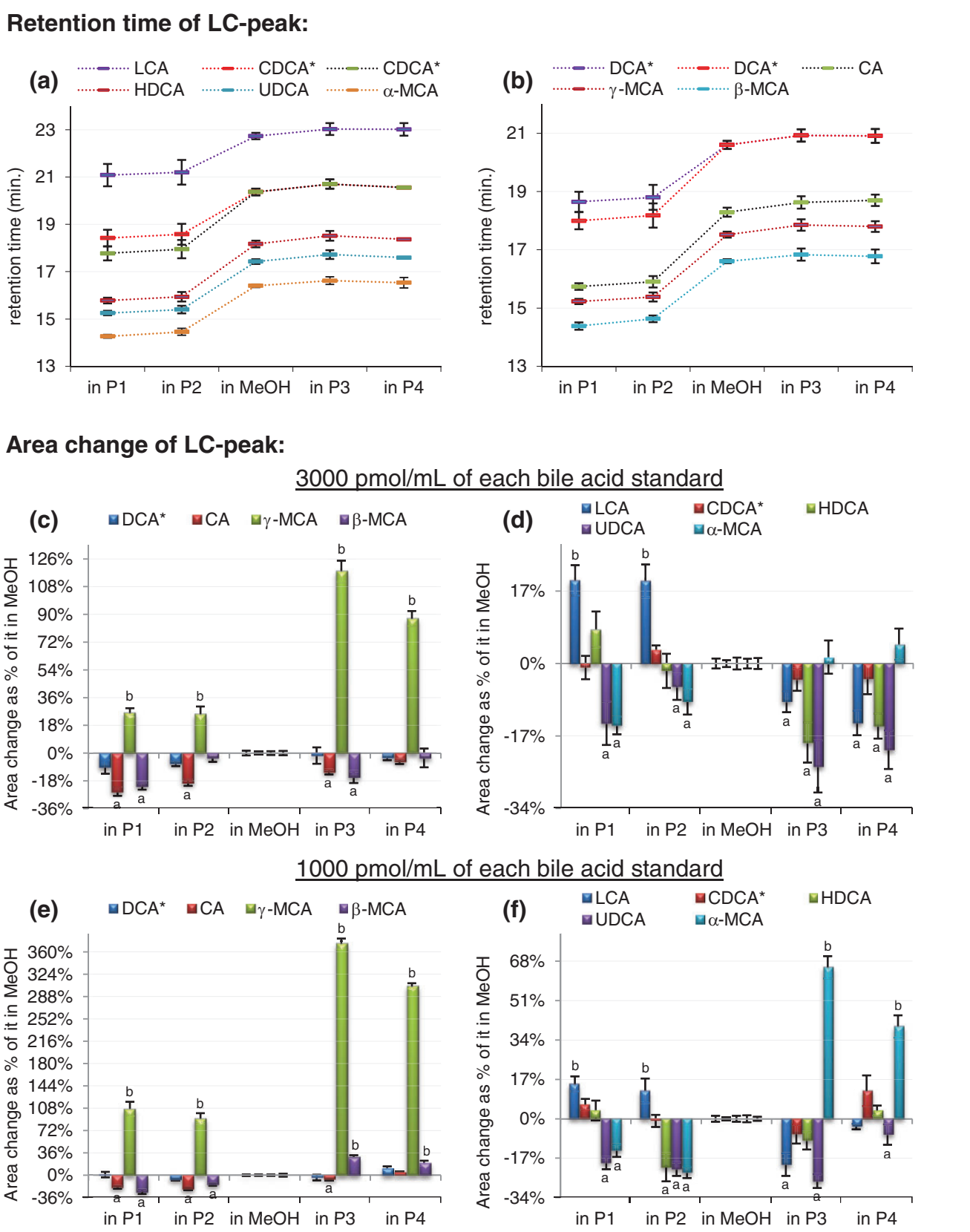

Figure 4 Matrix effects on unconjugated bile acids. (A), (B) Matrix effects on Rt. of LC-peaks. (C)–(F), matrix effects on peak area. *For the DCA and CDCA, one compound yielded two LC-peaks in P1 and P2. The areas used for DCA and CDCA in (C)–(F) were sum of its two peak areas, respectively. The area ratios of two DCA peaks in sum area were: (C), 1.00/0.65 ± 0.02 in P1 and 1.00/0.25 ± 0.06 in P2; (E), 1.00/0.55 ± 0.08 in P1 and 1.00/0.25 ± 0.02 in P2. The area ratios of two CDCA peaks in sum area were: (D), 1.00/0.71 ± 0.05 in P1 and 1.00/0.31 ± 0.03 in P2; (F), 1.00/0.52 ± 0.04 in P1 and 1.00/0.29 ± 0.02 in P2. The CDCA and DCA gave a single peak for one compound in P3 and P4. For the statistical analysis of Rt. for bile acid peaks, in P1 and P2 versus in MeOH: all are significantly different ($P < 0.001$); in P3 and P4 versus in MeOH: no significant matrix effect on bile acids was detected. For the areas of the peaks in P1–P4 versus in MeOH, letter ''a'' indicated a significant decrease and ''b'' indicated a significant increase ($P < 0.001$). (A color version of this figure is available in the online journal.)

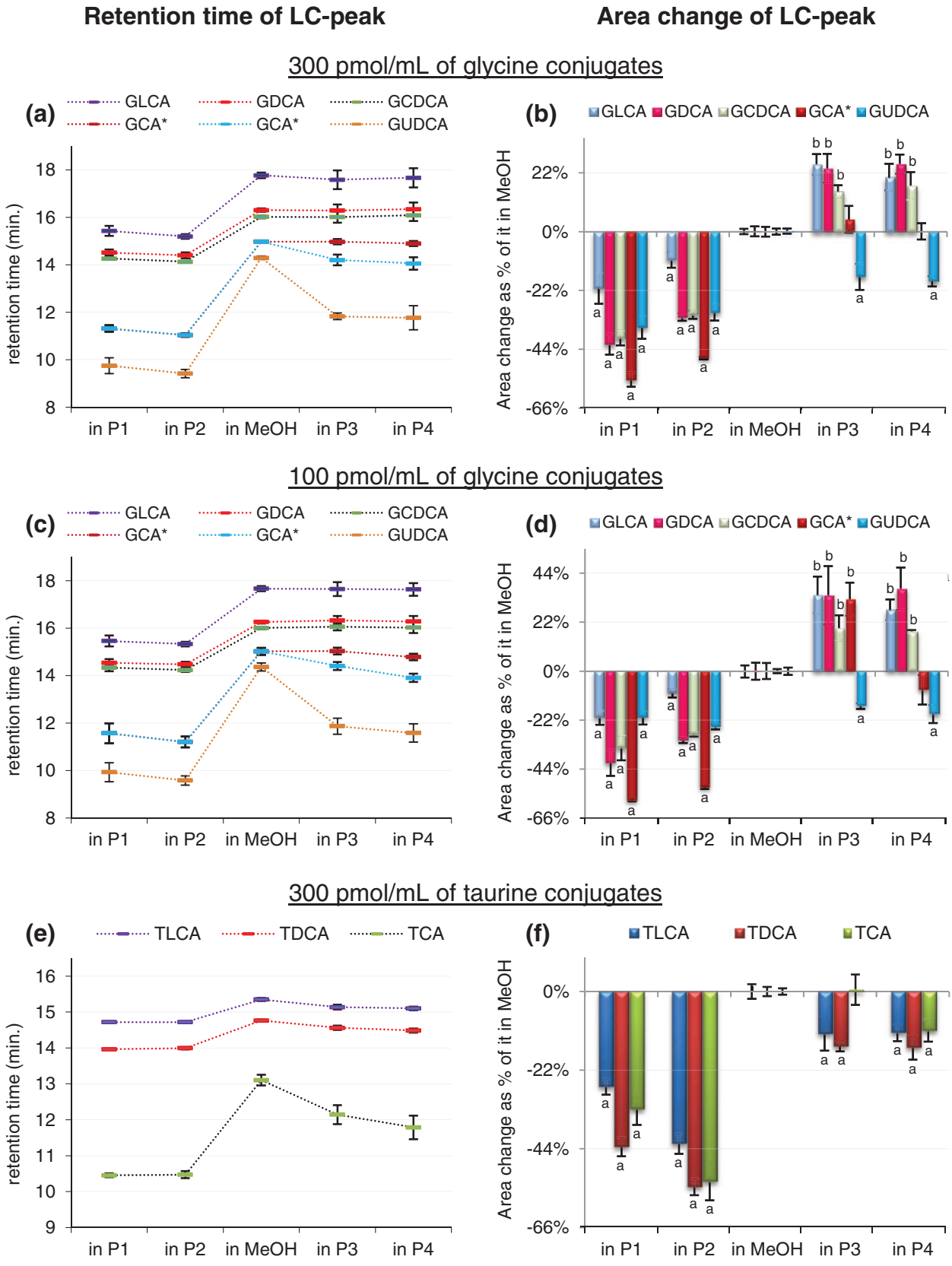

Figure 5 Matrix effects on conjugated bile acids. (A, C, and E) Matrix effects on Rt. of LC-peaks. (B, D, and F) Matrix effects on peak area. *GCA yielded two LC-peaks in P3 and P4. The area used for GCA in (B) and (D) was sum of its two peak areas. The area ratio of two GCA peaks in sum area was (B), 1.00/0.63 ± 0.04 in P3 and 1.00/0.98 ± 0.10 in P4; (D), 1.00/0.65 ± 0.01 in P3 and 1.00/215 ± 0.12 in P4. The GCA gave a single peak in P1 and P2. For the statistical analysis of Rt. for bile acid peaks, in P1 and P2 versus in MeOH: all are significantly different ($P < 0.001$); in P3 and P4 versus in MeOH: GUDCA, TCA, TDCA, and TLCA are significantly different ($P < 0.001$), no significant different was detected for GDCA, GCDCA, and GLCA, and only one of the GCA peaks was decreased significantly ($P < 0.001$). For the areas of the peaks in P1–P4 versus in MeOH, letter ''a'' indicated a significant decrease and ''b'' indicated a significant increase ($P < 0.001$). (A color version of this figure is available in the online journal.)

not show clearly significant effect on that of CDCA and HDCA (Figure 4C and D). For the conjugated bile acids, the matrix components in P1 and P2 decreased the LC-peak areas of all eight of the conjugates. However, the matrix effects of P3 and P4 caused significant increases in the peak areas of GCDCA, GDCA, and GLCA, and decreases in that of GUDCA, TLCA, and TDCA. We note that because there were no endogenous GCDCA, GDCA, and GLCA detected in P3 and P4 (Figure 3), it follows that the increases in the areas of the LC-peaks of GCDCA, GDCA, and GLCA were entirely due to the matrix effects of P3 and P4. On the other hand, the increases in the peak areas of γ-MCA in all urine samples (P1, P2, P3, and P4) relative to that of γ-MCA in pure methanol were due to the presence of the endogenous γ-MCA in urine (Figure 2), and the actual matrix effect of urine on the exogenous γ-MCA was to decrease the area of the corresponding LC-peak. For example, the area of the LC-peak of γ-MCA in P3 plus 3000 pmol/mL of exogenous γ-MCA sample was 118 ± 7% of the area of the LC-peak of 3000 pmol/mL of γ-MCA standard in pure methanol (i.e. without matrix components). The area of the LC-peak of γ-MCA in P3 plus 1000 pmol/mL of γ-MCA standard was 373 ± 9% of that of 1000 pmol/mL of γ-MCA standard in pure methanol (i.e. without matrix components). Hence, while matrix effect decreased the area of the LC-peak of the exogenous γ-MCA in the biological sample, the area of the LC-peak of endogenous γ-MCA did not decrease because it was endogenous to the biological sample and its area was already affected by its co-existing matrix components (Figure 2). This suggests that compounds endogenous to the biological sample must be considered when matrix effects are quantitatively investigated.

For quantification of analyte in biological sample, many strategies have been tried to minimize matrix effects, including more extensive sample preparation and optimization of chromatographic conditions.[4] The only accurate method for quantification in this manner is addition of stable-isotope-labeled internal standards (SIL-ISTDs). SIL-ISTDs are routinely used to mitigate matrix effects during LC-MS/MS analysis.[4] However, labeled metabolites are not readily available and there are a number of limitations to the use of SIL-ISTDs.[1] In a conventional procedure of quantification by LC/MS/MS, a SIL-ISTD is used for quantification of its analogues or a group of related compounds. The procedure may lead to an inaccurate quantitative result because the areas of different compounds are significantly variable under the matrix effects even when their chemical structures are very closely related (Figures 4 and 5). A matrix-matched calibration curve can be established by analyte's standard that compensates for matrix effects for quantification of the endogenous analyte. A matrix-matched calibration curve can be established by data from LC-MS/MS analyses of the biological samples with or without the analyte's standard. It should be noted that the matrix effects could be masked if the concentration of standard in the biological sample is too high. This method of quantification of analyte in biological sample is more practical than the method using SIL-ISTDs.

Discussion

Matrix effects become a major problem for the analysis of biological samples using LC-MS/MS.[4] All matrix effects which have been previously reported are on the ionization of the analyte which can lead to errors in sample quantitation. The accepted matrix effect mechanism is that matrix effects arise at the interface (ion source) between the LC system and the MS system, and anything that interferes with either the charging or the desolvation of the analyte will produce a matrix effect to change the peak area.[4] However, in the current study, matrix effects were observed on both peak area and LC behavior including peak shape and Rt. One possible explanation for this type of matrix effect is that some matrix components were loosely bonded to the analyte during the analysis, which affected the LC behavior of the analyte on the chromatography column, and interfered with ionization of the analyte at the MS ion source. Also, it is possible that co-eluted matrix components alter the pH in the mobile phase in the column when analytes interact with the stationary phase of the column. The results indicate that matrix effects cannot only adversely affect quantification of analytes, but also misguide the detection of analytes when the LC behavior of its corresponding standard is used. Due to the Rt. shift of the analyte from matrix effects, a false negative error would occur whereby the endogenous analyte would be not appear at the Rt. of its corresponding standard. Furthermore, matrix effects resulting in two peaks for one analyte would cause the analyte to be erroneously identified as its two isomers because of the different Rt. of LC-peak and same MS data. This is the first characterization of this kind of matrix effect in the literature.

In the last 10 years, the field of metabolomics has seen rapid advances in the development of automation software for identification and quantification of metabolites in biological samples. On the basis of the findings in the present study, matrix effects in LC-MS/MS analysis should be examined and accounted for before LC-MS/MS data are analyzed by automation software where identification of a metabolite is based on its exact molecular weight and retention time. The results also have significant implications in areas such as pharmacokinetics, drug development, and sports drug testing, especially when automation software is used in detecting and quantitating analytes of interest in biological samples.

Authors' contributions: All authors contributed to designing the study, doing the experiments, analysing the results, and writing the paper.

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