Abstract
When analyzing trace components *in vivo*, it is important to truly capture the target molecules, and even if advanced separation analysis methods are used, it is necessary to perform sufficient pretreatment. In recent years, chromatography and mass spectrometry have become commonly used for the analysis of biological components. Rapid development in both of these technologies has made it possible to capture previously undocumented phenomena. We have comprehensively analyzed cholesterol metabolites, including bile acids and their conjugated metabolites, *in vivo*, using both technologies. In addition, we have applied these techniques not only to small molecules *in vivo* but also to the analysis of biopolymers interacting with them. These results have contributed to the development of drug therapy, diagnosis, and other medical procedures, and we introduce some of these examples in this paper.

Keywords: Bile acid; Cholesterol metabolite; Drug; Protein; Liquid chromatography/tandem mass spectrometry

1. Introduction

Bile acids, the main components of bile, which is secreted after meals, play an important role in fat uptake *via* formation of micelles mixed with lecithin and other phospholipids. They have a 5β-steroid skeleton, a steroid skeleton in which the A-B ring bond is cis-type. The β-side of bile acids is highly hydrophobic, but hydrophilic functional groups such as hydroxy groups and carboxy groups exist on the α-side. Due to these structural features, bile acids show amphiphilicity. In humans, the primary bile acids are 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid, CA) and 3α,7α-dihydroxy-5β-cholan-24-oic acid (chenodeoxycholic acid, CDCA), which are biosynthesized from cholesterol in the liver. The main secondary bile acids are 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid, DCA), 3α-hydroxy-5β-cholan-24-oic acid (lithocholic acid, LCA), and 3α,7β-dihydroxy-5β-cholan-24-oic acid (ursodeoxycholic acid, UDCA), which are produced from primary bile acids by the action of several enzymes in intestinal bacteria (Fig. 1A). Bile acids are localized in the enterohepatic circulatory system by an efficient transport mechanism and usually only trace amounts are found in circulating blood.

In 1999, Makishima *et al.* [1] and Parks *et al.* [2] found that bile acids are native ligands of a nuclear farnesoid X receptor, FXR, and that the binding of bile acids to FXR is the starting point for a negative feedback mechanism via suppression of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme for bile acid biosynthesis. FXR has also been known to modulate the bile acid pool through altering the expression of membrane transporters [3]. Moreover, the G-protein-coupled receptor TGR5 has been identified as a membrane-type receptor for bile acids [4,5], and Watanabe *et al.* [6] have reported that the binding of bile acids with TGR5 induces the cyclic-AMP-dependent thyroid hormone activating enzyme, type 2 iodothyronine deiodinase. The signal-regulating functions of bile acids related to the regulation of the bile acid pool, FXR, and TGR5 have been attracting attention as potential drug discovery targets for the treatment of metabolic diseases such as obesity, type 2 diabetes, and hypertriglyceridemia [7].

In the first half of this review, we focus on the development of highly sensitive and reliable quantitative determination methods for bile acids and their metabolites, and their application in clinical practice. In addition, the precise analysis of proteins with which bile acids interact is also described. In the latter part of this review, we develop focused metabolome analysis methods for conjugated...
Chromatography

cholesterol metabolites based on information obtained by the development of a conjugated bile acid analysis method, and develop a noninvasive diagnostic method for Niemann-Pick disease type C (NPC). In addition, we also introduce some examples of the application of liquid chromatography/tandem mass spectrometry (LC/MS/MS) to therapeutic drug monitoring (TDM).

(A) Bile acids

| Compound | Structure |
|----------|-----------|
| Cholic acid (CA) | OH α-OH OH OH |
| Chenodeoxycholic acid (CDCA) | OH α-OH H OH |
| Deoxycholic acid (DCA) | OH H H OH |
| Lithocholic acid (LCA) | OH H H OH |
| Ursodeoxycholic acid (UDCA) | OH β-OH H OH |

(B) Conjugates

| Compound | Structure |
|----------|-----------|
| Glycine conjugate | NHCH\_2COOH |
| Taurine conjugate | NHCH\_2CH\_2SOH |
| 3-Sulfate | OSOH |
| 3-Glucuronide | NHCH\_2COOH |
| 3-Glucoside | OH |
| 7-N-Acetylgalactosaminide | OH |
| 24-Glucuronide | OH |
| 24-Galactoside | OH |

Fig. 1. Structures of bile acids and their conjugates.

2. Analysis of bile acids and their conjugates

Bile acids are converted into various conjugated metabolites in the body (Fig. 1B). The hydroxy group at the C-3α position undergoes sulfation [8,9], glucuronidation [10-12], and glucosidation [13,14]. The 7β-hydroxy group of UDCA is conjugated to N-acetylgalactosamine [15,16], and the conjugates have been found in the patient urine after large amounts of UDCA was administered for the treatment of primary biliary cirrhosis [17]. The carboxy group at the C-24 position is usually conjugated with glycine or taurine, but our research group found that bile acid acyl glucuronides are present in human urine [18,19].

The bile acid conjugates described above all have ionic functional groups, but when neutral sugars are bonded to the carboxy group at the C-24 position, the charge of the entire molecule is substantially lost. Because electrospray ionization (ESI) is not suitable for the ionization of electrically neutral molecules with only hydroxy groups, it is difficult to analyze these neutral molecules with high sensitivity using LC/ESI-MS/MS. We found that adding an anion in the gas phase to neutral molecules which have only hydroxy groups permitted analysis with high sensitivity using LC-MS/MS equipped with atmospheric pressure chemical ionization [20]. Bile acid acylglucosides in human urine were examined using this method, and two peaks different from the standard were found. As a result of structural analysis, they were found to be CA and DCA acyl galactosides [21].

When analyzing bile acid 3-sulfates in human urine using LC-ESI-MS/MS, we found an unknown but relatively intense peak between UDCA 3-S and CDCA 3-S [22]. Nambara and Goto [23] have reported relative retention factors of dihydroxylated bile acids under the isocratic elution condition in detail. According to their description, the molecules that are likely to elute between UDCA and CDCA are 3α,12β-form, 3β,7α-form, and 3β,12α-form. It was suggested that the unknown peak is the sulfate conjugate of 3β,12α-dihydroxy-5β-cholan-24-oic acid, 3-isoDCA (3β,12α-form), since the retention time of the compound liberated by solvolysis was consistent with that of 3-isoDCA. By synthesizing a standard of 3-isoDCA 3-sulfate and comparing retention times, it was confirmed that the unknown peak was the 3-sulfate conjugate of 3-isoDCA. Interestingly, 3-isoDCA, in which the carboxy group at the C-24 position was conjugated with glycine or taurine, was not present in the blood and urine of healthy adults (Table 1). Conversely, despite the absence of sulfates of other free bile acids, there was a large amount of 3-isoDCA 3-sulfate in human urine. Goto and colleagues [24] reported the presence of an enzyme that reduces 3-oxo bile acids to 3β-hydroxylated bile acids in human erythrocytes. The presence of a large amount of 3-isoDCA 3-sulfate in the urine may suggest the existence of DCA’s extracorporeal pathway, which has been identified for some time as a promoter of colorectal cancer.

Since Weil [25] suggested that taurocholic acid (TCA) causes significant demyelination in vitro and some bile acids may act as a natural demyelinating agent, it has been thought that bile acids cannot exist as it is in the brain. Conversely, if bile acids are present in the brain, we considered that bile acids are present in association with proteins to prevent demyelination of neurons. Therefore, bile acids in the rat brain were analyzed using a special extraction method that dissociates reversible protein bindings. As a result, there was an amount of free bile acid present in rat brain tissue that was not considered to be derived from capillary blood; CDCA was the most abundant of these [26].
Early in the morning, we decided to identify the 3-epimer binding to CDCA, we decided to construct a selective extraction method for small molecule-binding proteins that could avoid contamination with nonspecific binding proteins [27]. In this method, a cleavable moiety that can be cleaved by a relaxed chemical reaction is introduced into a linker between ligands and a gel carrier, as shown in Fig. 2. The target protein is bound to the ligand, followed by washing, and the linker is cleaved to recover the bound protein. When CDCA binding proteins were extracted by mixing a cytoplasmic fraction prepared from rat brain tissue with the cleavable affinity gel, in addition to tubulin, actin, and 14-3-3 proteinζ, abundant quantities of growth hormone from the pituitary were extracted [28]. Although it cannot be said that the specificity is high, it was found that CDCA and growth hormone bind reversibly. This has been demonstrated through an affinity labeling method using bile acid acyl adenylate as the labeling reagent (Fig. 3) [29]. It is quite interesting to consider the relationship of bile acids with various hormones secreted from the anterior pituitary.

| Table 1. Concentration of bile acid 3-sulfates in 14 healthy volunteers. |
|------------------|---------------|-------|
| **Bile acid 3-sulfate (ng) / creatinine (mg)** |
| **Subject** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** | **M** | **N** | **Ave. ± SD** |
| (a) Unconjugates |
| CA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| UDCA-3S | 42 | 201 | NQ | 79.0 | 98.9 | 145 | 36.9 | 465 | 55.2 | 76.6 | 81.9 | 90.1 | 48.9 | 110 | ± 115 |
| IsoDCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| CDCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| LCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| (b) Glyco-conjugates |
| CA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| UDCA-3S | 66.8 | NQ | 112 | 52.1 | 115 | 96.3 | 43.1 | 96.4 | 35.7 | 92.8 | NQ | 294 | 88.8 | 23.9 | 84.2 | ± 69 |
| IsoDCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| CDCA-3S | 107 | 114 | 367 | 120 | 451 | 372 | 130 | 154 | 343 | 77.3 | 411 | 412 | 172 | 240 | ± 140 |
| DCA-3S | 529 | 294 | 109 | 376 | 241 | 111 | 401 | 483 | 535 | 917 | 139 | 351 | ± 220 |
| LCA-3S | 468 | 205 | NQ | 707 | 134 | 372 | 311 | 190 | 33.9 | 76.6 | 155 | 210 | 986 |
| (c) Tauro-conjugates |
| CA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| UDCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| IsoDCA-3S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| CDCA-3S | 38.0 | 43.1 | 18.7 | 27.8 | NQ | 38.9 | 47.8 | 20.6 | 34.0 | 51.2 | 39.8 | 30.5 | ± 14 |
| DCA-3S | 116 | 120 | NQ | 88.7 | 134 | 19.3 | 14.3 | 18.2 | ± 22 |
| LCA-3S | 334 | 106 | 60.3 | 61.7 | 157 | 23.2 | 44.4 | 110 | 126 | ± 88 |
| Total | 1801 | 1040 | 630 | 899 | 1058 | 580 | 1762 | 974 | 1709 | 2024 |

NQ; less than the limit of quantitation, -; not detected.
3. Protein adduct production through chemically active intermediates

As mentioned earlier, there are bile acid glucuronides in the human body produced via the 3α-hydroxy group and the carboxy group at the C24-position. The conversion of bile acids to 24-glucuronides has been reported predominantly progresses in human liver [19,30]. Acyl glucuronides are chemically active and covalently bind to amines such as proteins [31-34]. For the irreversible complex formation of acyl glucuronides and proteins, amino groups of the proteins may react with acyl groups to form amides, and Amadori rearrangement products may be formed through condensation with reducing sugars. We found that the enzyme involved in the production of bile acid acyl glucuronides was inhibited through negative feedback by an increase in the amount of product [35]. In addition, it was found that glycine- and taurine-conjugated bile acids also inhibited the enzymatic reaction, and that the bile acids themselves inhibited the formation of irreversible protein complexes [36]. Our research has also found that acyl glucuronides and amino acid conjugates of bile acids inhibit the enzymatic reactions involved in the production of acyl glucuronides by propionate-based anti-inflammatory drugs [36].

There is a bile acid metabolite that is more reactive than the acyl glucuronides. Amino acid conjugation of bile acids proceeds by a two-step reaction, first through the formation of CoA thioester by bile acid-CoA ligase and then amino acid conjugation by amino acid N-acyltransferase. ATP is necessary for the first step of the reaction, which means that an exchange reaction between AMP and CoA occurs after the bile acid is converted to acyl adenylate (Fig. 3A) [37]. Bile acid acyl adenylates are chemically very active and readily reacts with amines to form amides [38]. In fact, taurine-conjugated bile acids are produced even if incubated with taurine without an enzyme. Moreover, the reaction is instantaneously completed. From this, it can be easily imagined that it would react with the protein to form a covalent adduct if bile acid acyl adenylate exists in the body. Interestingly, when bile acid acyl adenylate was nonenzymatically mixed with lysozyme, it was found that the reaction readily progressed and bound bile acid to the lysine residue located sterically outside of lysozyme. We confirmed whether histone H3, which is one of the constituent proteins of the nucleosome and has a flexible tail rich in basicity, was irreversibly modified by bile acid acyl adenylate. As a result, binding of a plurality of lysine residues on the N-terminal tail of histone H3 was observed when a large amount of acyl adenylate was present on the protein [39]. On the other hand, it was found that when the molar ratio was lowered, the lysine residue at the 4th position from the N-terminus was most reactive. If depletion of CoA occurs in the body, there is a possibility that acyl adenylate may be liberated without completing the reaction of bile acid CoA ligase, and it is conceivable that such irreversible protein adducts are produced as a result.

Fig. 2. Design of cleavable affinity gel for specific extraction of small molecule-binding proteins.

![Design of cleavable affinity gel for specific extraction of small molecule-binding proteins.](image)

Fig. 3. Affinity labeling for analysis of a small molecule-binding site in protein. (A) Acyl adenylate as an affinity labeling reagent. (B) Analytical method for determination of a small molecule-binding site by affinity labeling.
It becomes possible to use bile acid acyl adenylate as an affinity labeling agent for analyzing the binding of a small molecule ligand to a protein by utilizing its high reactivity (Fig. 3A). First, a small molecule such as a ligand, is converted to an acyl adenylate derivative, which is then incubated with a binding protein to invade the binding site. This activates the reaction to immobilize the ligand molecule in the vicinity of the binding site. Then, ligand-protein adducts are enzymatically digested, and examined by mass spectrometry to analyze the ligand binding site (Fig. 3B). An anti-DCA monoclonal antibody was used as a model protein and analysis of the antibody recognition site by affinity labeling was attempted using DCA acyl adenylate [40]. After the reaction, peptides to which DCA was irreversibly bound were extracted from the peptide mixture using an affinity gel on which an anti-DCA monoclonal antibody was immobilized and analyzed by LC/ESI-MS/MS. DCA was bound to lysine74 residue sandwiched between complementarity-determining region (CDR) 2 and CDR3 of the heavy chain variable region and was found to be bound to the entrance of the pocket of the antigen recognition site. This revealed that acyl adenylate can function sufficiently as an affinity labeling agent, and this technique was used for analyzing a binding site for CDCA to growth hormone in the previous section.

4. Focused metabolomics of conjugated cholesterol metabolites

As mentioned earlier, bile acids undergo various conjugative metabolic processes. The conjugated site is chemically unstable and easily cleaved with low energy collision-induced dissociation (CID). We attempted to construct a focused metabolome analysis method utilizing such properties of bile acid conjugates. First, the cleavage behavior of each bile acid conjugate at low energy CID was investigated [41]. As a result, it was found that characteristic product ions or neutral loss occurred based on the conjugation type (Table 2). Focused metabolome analysis conditions of each conjugate were examined on the basis of these results and validity was verified using various preparations [42].

Table 2. Estimation of conjugation type by product ions and neutral losses observed in low-energy CID.

| Product ion (m/z) | Neutral loss (Da) | Conjugation type                |
|------------------|-------------------|---------------------------------|
| m/z 74           |                   |                                 |
| m/z 124          |                   |                                 |
| m/z 107, m/z 80  |                   |                                 |
| m/z 97, m/z 80   | 98, 80            | Sulfate (aliphatic alcohol)     |
| m/z 97, m/z 74   | 173, 75           | Glycine-conjugated sulfate      |
| m/z 124, m/z 97  | 152, 98           | Taurine-conjugated sulfate      |
| m/z 80           | 80                | Sulfate (phenolic alcohol)      |
| m/z 161, m/z 113 | 162               | Acyl galactoside                |
| m/z 175 (strong) | 176               | Acyl glucuronide                |
| m/z 175, m/z 113 | 176 (strong)      | Glucuronide (phenolic ether)    |
| m/z 175 (weak)   | 176               | Glucuronide (aliphatic ether)   |
| m/z 113          |                   |                                 |
| m/z 202, m/z 100 | 221, 203          | N-Acetylglucosamine conjugate   |
|                  |                   | 221                             | N-Acetylglucosamine conjugate (A-structure) |

The underlined part represents a strong feature for estimation.

Fig. 4. Total ion current chromatogram of a mixture of authentic conjugated cholesterol metabolites. Conditions: Apparatus, an API 5000 mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA, USA) coupled with a Nanospace SI-2 LC system (Shiseido Co., Ltd., Tokyo); ionspray voltage, −4500 V; declustering potential, −80 V; turbo spray gas probe temperature, 700°C; curtain gas (N2), 25 units; ion source gas1 (N2), 40 units; ion source gas2 (N2), 60 units; collision gas (N2), 6 units; collision voltage, −60 V; trapping column, Shim-pack MAYI-C8 (4.6 mm i.d. × 10 mm, Shimadzu, Corp., Kyoto); analytical column, YMC-Pack Pro C18 (5 μm, 2.0 mm i.d. × 250 mm, YMC Co., Ltd., Kyoto); mobile phase for trapping, a mixture of 20 mmol/L ammonium acetate solution (pH 7.0) and methanol (9:1, v/v); flow rate for trapping, 1 mL/min; mobile phase for a linear gradient elution, A, 20 mmol/L ammonium acetate solution (pH 7.0) and methanol (9:1, v/v), B, (1:19, v/v); gradient program, the rate of solvent B was linearly changed from 50% to 80% over 120 min and then it was kept at 80% for 20 min and switched to 50% at 140 min and kept for 40 min; scan range, m/z 200 to 900.
NPC is a lysosomal disease in which cholesterol and glycolipids abnormally accumulate in cells. This disease is based on mutations in the \( NPC1 \) and \( NPC2 \) genes [43-46]. Symptoms manifested by the onset of the disease are variable based on age at presentation; hepatosplenomegaly is more common in onset during the neonatal period, whereas various central nervous symptoms are seen in patients who develop it in adulthood [45-47]. One of the characteristics of this disease is its diversity of symptoms, and therefore diagnosis is very difficult [45,46]. Currently a method of culturing fibroblasts after skin biopsy and diagnosis by Giemsa staining or filipin staining along with a gene analysis method are used. This is not a good definitive diagnostic method as it is invasive and lacks diagnostic accuracy [45,46]. From this point of view, we began to develop a diagnostic method for NPC with low invasiveness.

![Fig. 5. Structures of 3β-sulfooxy-7β-N-acetylglucosaminyld-5-cholen-24-oic acid and its glycine and taurine conjugates.](image)

First, analysis of the sulfate-conjugated cholesterol metabolite group (precursor ion scan of \( m/z \) 97) (Fig. 4) was performed using NPC patient urine, and the presence of characteristic peaks in disease was investigated [42]. As a control, urine from 3β-hydroxysteroid dehydrogenase (3β-HSD) deficient patients, healthy adults, and healthy children were similarly analyzed and compared with the results of NPC patients. In healthy adults and healthy children, few peaks were observed, but multiple peaks were detected in NPC patients and 3β-HSD deficient patient urine. Several peaks detected in NPC patients and not seen in 3β-HSD deficient patients were examined and were consistent with the previously reported metabolites [48] of 3β-sulfooxy-7β-N-acetylglucosaminyld-5-cholen-24-oic acid (SNAG-\( \Delta^5 \)-CA) and its glycine (SNAG-\( \Delta^5 \)-CG) and taurine conjugates (SNAG-\( \Delta^5 \)-CT) (Fig. 5). Next, we designed a quantitative determination method and measured urinary concentrations of these compounds in NPC patients. It was found that concentrations in the urine of newborns with NPC were several hundred times higher than those of healthy adults [49]. Current diagnostic practice is now to collect and analyze urine from patients diagnosed with NPC, as the data suggests that these three kinds of metabolites are useful as diagnostic markers of NPC.

### 5. Drug analysis for therapeutic drug monitoring

To individualize patient drug therapy, monitoring of factors related to the therapeutic effects and side effects of the drugs is very important. The concentration of drug in the blood is an especially important factor, as shown in recent studies, which demonstrate the effectiveness of TDM for several oral anticancer agents [50,51]. Usually, immunoassay or chromatography is used to measure plasma drug concentration. Although the former is easy to operate and produces results quickly, it is expensive and the quality of the antibody used tends to influence the results. On the other hand, the latter is difficult to operate and takes time, but it is advantageous for simultaneous analysis of other compounds such as metabolites. Recently, LC/ESI-MS/MS has commonly been used for measurement of plasma drug concentrations; however, the quality of the data has sometimes been compromised when priority has been given to rapidity and convenience. We experienced variations in measured values due to the matrix effect in plasma concentration measurements of sirolimus (Fig. 6), an inhibitor of the mammalian target of rapamycin [52]. The cause of this matrix effect was the large amount of lysosphospholipids coexisting in the sample. Highly accurate measurement became possible by adding a pretreatment step, which removed them [53]. In order to realize personalized medicine, it is important to achieve accurate measurements of plasma drug concentrations, and even if LC/MS/MS with high selectivity is used, ensure that the necessary pretreatment operations are performed.

![Fig. 6. Structure of sirolimus.](image)
For some drugs, the quantification of the metabolites, as well as the administered drug, may be necessary for controlling the therapeutic effects and adverse events [54,55]. The ionization used in LC/MS/MS depends on the physical and chemical properties of the analyte, and ionization can be greatly influenced by a small structural change. Therefore, it may occasionally not be possible to simultaneously quantify the unchanged drug and metabolites. In such a case, it is usual to analyze each by dividing the analysis into two, but methods for simultaneous quantification have been also reported. Although the strongest product ion is commonly used for the transition of selected reaction monitoring, and by using another product ion, the amount of ions reaching the detector can be adjusted [56,57]. However, in this method, since the adjustment of ion quantity reached to the detector depends on the production ratio of product ions, its success depends greatly on the chemical structure of the analyte. On the other hand, when the isotope of the precursor ion is monitored, the amount of ions to be introduced into the collision chamber can be adjusted, and it has been reported that this greatly improves linearity [58,59]. Although this method can theoretically adjust the amount of ions, in reality since the ion quantity is determined by the element composition, arbitrary control is difficult. We have proposed a linearity control technique using in-source CID [60,61]. In this case, the amount of ions to be introduced into the mass spectrometer can be arbitrarily adjusted, and linearity can be secured more broadly (Fig. 7). This method is applicable to the simultaneous analysis of drugs and metabolites in human plasma [62], and recently it has been applied to the simultaneous analysis of multiple oral anticancer drugs in plasma from renal cell carcinoma patients [63].

6. Conclusion

In this review, we introduced high sensitivity and high precision separation analysis methods for cholesterol metabolites, mainly bile acids, which we constructed using chromatography and mass spectrometry. All of the analytical methods discussed in this review are applicable to the measurement of trace components in complicated matrices such as biological samples and have also made clear the existence of new molecules in vivo. In addition, highly accurate analytical methods that make use of advanced separation analysis methods can be applied not only to small molecules but also to the analysis of biopolymers that interact with them, contributing to the functional analysis of small molecules in vivo. In addition, such a high precision separation analysis method is also an important means for TDM in clinical practice, which greatly contributes to individual optimization of drug therapy and efficiency of medical care. Advances in separation analysis methods are indispensable for the development of clinical chemistry research, and further development of this research field is expected in the future.

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Fig. 7. Comparison of linear regression lines of 2'-deoxyuridine under control conditions and 30 V lower declustering potential conditions using in-source CID.
Chromatography

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