Full $^1$H and $^{13}$C NMR spectral assignment of conjugated valerolactone metabolites isolated from urine of black tea consumers by means of SPE-prepLC–MS–LC–MS-NMR

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Abstract
The health benefits of black tea have been linked to polyphenol metabolites that target specific modes of action in the human body. A major bottleneck in unravelling the underlying mechanisms is the preparative isolation of these metabolites, which hampers their structural elucidation and assessment of in vitro bioactivity. A solid phase extraction (SPE)-preparative liquid chromatography (prepLC)–MS–LC–MS-NMR workflow was implemented for preparative isolation of conjugated valerolactone metabolites of catechin-based polyphenols from urine of black tea consumers. First, the urine was cleaned and preconcentrated using an SPE method. Subsequently, the clean urine concentrate was injected on a preparative LC column, and conjugated valerolactones were obtained by MS-guided collection. Reconstituted fractions were further separated on an analytical LC column, and valerolactone fractions were collected in an MS-guided manner. These were reconstituted in methanol-$d_4$ and identified and quantified using 1D and 2D homo- and heteronuclear NMR experiments (at a field strength of 14.1 T), in combination with mass spectrometry. This resulted in the full spectral $^1$H and $^{13}$C NMR assignments of five conjugated valerolactones. These metabolites were collected in quantities of 8–160 μg and purities of 70–91%. The SPE-prepLC–MS–LC–MS-NMR workflow is suitable for isolating metabolites that occur at sub-μM concentrations in a complex biofluid such as urine. The workflow also provides an alternative for cumbersome and expensive de novo synthesis of tea metabolites for testing in bioactivity assays or for use as authentic analytical standards for quantification by mass spectrometry.

KEYWORDS
Polyphenols, SPE, tea, urine, valerolactones

ABBREVIATIONS: MSI, metabolite standards initiative; prepLC, preparative liquid chromatography; SPE, solid phase extraction
1 | INTRODUCTION

Tea is one of the most frequently consumed beverages in the world, and its consumption has been linked to cardiovascular health benefits, in particular to the prevention of stroke.\textsuperscript{[1–3]} The health benefits of tea have been linked to the presence of polyphenols,\textsuperscript{[4]} which are phytochemicals that share the motif of an aromatic ring with two or more hydroxyl groups. The mechanism of their direct antioxidant activity in the body has been abandoned, instead more specific and subtle effects of polyphenols and their metabolites have been proposed.\textsuperscript{[5,6]} It is now well appreciated that direct bioavailability of tea polyphenols is low, but their metabolites enter circulation at significant concentrations, in particular those originating from gut microbial catabolism.\textsuperscript{[7]} These catabolites can undergo different conjugations by the human host, which adds significant compositional diversity to the circulating metabolites.\textsuperscript{[8]} The earliest products from black tea polyphenol catabolism are the valerolactones, which are the results of catechin ring scissions.\textsuperscript{[4,9]} The valerolactones undergo extensive conjugation by host metabolism and sustained circulation at high systemic concentrations has been observed.\textsuperscript{[10]} Specific interactions of conjugated valerolactones at organ level have been proposed as the underlying mechanisms for the benefits of tea.\textsuperscript{[4]} In our recent collaborative work, we carried out large scale identification, isolation, and quantification of the circulating metabolites of black tea polyphenols.\textsuperscript{[11]} By hyphenation of LC, SPE, NMR, and MS, the identification of a large number of conjugated valerolactones could be achieved in both urine\textsuperscript{[12]} and plasma.\textsuperscript{[4]} These isolated fractions contained only small (μg scale) amounts of conjugated valerolactones in the presence of impurities. This hampered full \(^1\)H NMR spectral assignments, mainly due to lack of both 2D NMR data and nonoverlapping, well-resolved spectra.\textsuperscript{[10]} Although these isolated metabolite aliquots allowed for use as authentic standards for quantification of tea polyphenol metabolites by LC–MS, they were of insufficient amounts to be used for in vitro testing of bioactivity. For this purpose a minimal amount of \(\approx 10~\mu g\) of purified metabolites is required,\textsuperscript{[4]} which can currently only be obtained by de novo synthesis.\textsuperscript{[13–15]} Although synthetic routes will yield ample material, they involve significant investments in time and money.

The limitations of the LC-SPE-NMR-MS workflow for providing sufficient amounts of metabolites prompted us to develop an extended hyphenated approach for preparative (\(\approx > 10~\mu g\)) isolation and full heteronuclear (\(^1\)H and \(^{13}\)C) spectral assignment of conjugated valerolactones. The feasibility of this approach was based on estimates that in urine of habitual tea drinkers conjugated valerolactones appear at micromolar concentrations.\textsuperscript{[12]} Our approach involves offline 2D LC runs and SPE clean up steps, which requires rational and systematic optimisation and reconciliation of chromatographic conditions. Here, the aim is to obtain sufficient quantities to allow for assignment of both \(^1\)H and \(^{13}\)C NMR resonances.

2 | MATERIALS AND METHODS

2.1 | Urine collection

Three habitual tea drinkers involved in the project switched to drinking a brew prepared from dry tea powder (Lipton Yellow Label) for one morning. The urine of the volunteers was collected over 18 hr and pooled into a single container. On the basis of compositional data on the dry tea powder the tea brew contained 60 mg/L of catechins. On the basis of the estimated consumption of the tea brew, the total catechin intake was estimated as 30–45 mg/day. Because the urine donation was for methodological purposes and did not serve medical scientific research, no ethical approval was required under Dutch law. The pH of the pooled urine was adjusted to 3, using hydrochloric acid. The pooled urine was aliquoted into 100-mL jars and stored in a freezer at \(-80^\circ C\) until further use.

2.2 | Urine clean up and preconcentration by means of solid phase extraction (SPE)

Urine samples were cleaned up and preconcentrated, using an SPE method.\textsuperscript{[16]} The SPE cartridges (3 cc Oasis HLB, Waters) were conditioned with 1.5 ml of methanol (LiChrosolv, Merck), followed by 3 ml of milliQ water (Millipore). Then, 3 ml of pooled urine was brought on the cartridge, and the unretained fraction was discarded. The cartridges were subsequently washed with 1.5 ml of milliQ water; this wash fraction was also discarded. To reduce the levels of background metabolites, the cartridge was washed with 1.5 ml of 10% of methanol in milliQ water (Elution 1). Subsequently, the cartridge was eluted with 1.5 ml of methanol (Elution 2). The latter was evaporated to dryness using an Eppendorf Concentrator Plus at room temperature. The dried fractions were stored in a freezer at \(-20^\circ C\) until further use. The described SPE method, except the drying step, can be done in full automation, using a Gerstel MPS robot. However, sample throughput is relatively low, as the robot cannot operate in parallel mode. In manual mode however, an analyst can prepare up to 20 samples simultaneously, but this
would require more hands-on work. Shortly before LC–MS analysis, the dried fractions were dissolved in 11% of acetonitrile in milliQ water containing 0.1% (v/v) of formic acid. This step resulted in a 20 times concentration of the urine.

2.3 | PrepLC–MS separation

Preparative LC separations were performed on an Agilent Technologies 1200 series LC instrument, connected to a Bruker MicrOTOF II mass spectrometer (with a sample split of 1:50, between MS and fraction collector). A Phenomenex Kinetex C-18 column (10 × 250 mm, 5 μm) was used for separation. The injection volume was 500 μl. The eluents consisted of 0.1% (v/v) formic acid in milliQ water (A) and 0.1% (v/v) of formic acid in acetonitrile (B). The applied gradient started at 11% B for 5 min, followed by a linear increase to 21.6% B in 55 min, followed by an increase to 100% B in 1 min. The gradient was held for 5 min and then returned in 0.1 min to the starting conditions (11% B). The column was kept at room temperature. The samples were placed in an Agilent Technologies 1200 series autosampler at 4°C. The mass spectrometer scanned an m/z range from 50 to 950 in ESI negative mode, at a rate of 1 Hz/scan. The ionisation settings used were: capillary voltage 3.4 kV, dry gas temperature 200°C, dry gas flow 8 L/min, nebuliser pressure 2 Bar.

2.4 | SPE of prepLC–MS fractions

The fractions were combined with 100 ml of milliQ water and brought onto a 3 cc Oasis HLB SPE cartridge (Waters). The unretained fraction was discarded. The cartridges were eluted using 1 ml of methanol and subsequently evaporated to dryness at room temperature under a gentle stream of nitrogen. The fractions were dissolved in 190 μl of methanol-d₄ (99.8%D, EurisoTop) and transferred to 3-mm NMR tubes using a glass Pasteur pipette.

2.5 | LC–MS fraction purification

Separations were performed on the LC system as described above. The column was changed to an analytical Phenomenex Luna C-18 column (4.6 × 250 mm, 5 μm). After the scout runs, the remainder of the dried fraction was dissolved in the eluent composition used at the start of each tailored method. The injection volume used was 50 μl. Fractions were collected manually and subsequently evaporated to dryness at room temperature under a gentle stream of nitrogen. The dried fractions were dissolved in 190 μl of methanol-d₄ (99.8%D, EurisoTop) and transferred to 3-mm NMR tubes for analysis. The LC–MS chromatograms are available upon request.

2.6 | NMR measurements

1D ¹H NMR spectra were recorded with the NOESYGPR1D pulse sequence on a Bruker Avance III 600 NMR spectrometer (14.1 T), equipped with a 5-mm cryo-probe. The probe was tuned to detect ¹H NMR resonances at 600.25 MHz. The internal probe temperature was set to 300 K. In total, 64 scans were collected in 64 k data points with a relaxation delay of 30 s, an acquisition time of 2.73 s and a mixing time of 150 ms. Low power water suppression (16 Hz) was applied for 1 s. The spectral width was 20 ppm. The spectra were processed in TopSpin software version 3.5 pl 1 (Bruker BioSpin GmbH, Germany). An exponential window function was applied to the free induction decay with a line-broadening factor of 0.3 Hz prior to the Fourier transformation. Manual phase and baseline correction was applied to all spectra. The spectra were referenced to the residual signal of methanol-d₄ at δ 3.3 ppm. Additionally, 2D ¹H - ¹H TOCSY spectra were recorded using the MLEVPHPR pulse sequence. The spectral widths were 20 ppm. Thirty-two scans in the direct dimension (2 k data points) and 128 increments in the indirect dimension were collected. The recycle delay was 1.5 s, and the mixing time was 45 ms. 2D ¹H - ¹³C HSQC spectra were recorded using the HSQCEtGPPRISp2.2 pulse sequence. The spectral widths were 12 (¹H dimension, 2 k data points) and 150 ppm (¹³C dimension). Sixty-four scans in the ¹H dimension and 128 increments in the ¹³C dimension were collected. The recycle delay was 1.5 s. Suppression of the solvent signal (methanol-d₄) was applied during the recycle delay. The spectra were referenced to the residual signal of methanol-d₄ at δ 3.3 ppm (¹H) and δ 50 ppm (¹³C). 2D ¹H - ¹³C HMBC spectra were recorded using the HMBCGPlPNDPQR pulse sequence. The spectral widths were 12 (¹H dimension, 2 k data points) and 200 ppm (¹³C dimension). Forty-eight scans in the ¹H dimension and 128 increments in the ¹³C dimension were collected. The recycle delay was 2 s. The long range coupling constant was set to 8 Hz. Suppression of the solvent signal (methanol-d₄) was applied during the recycle delay. The spectra were referenced to the residual signal of methanol-d₄ at δ 3.3 ppm (¹H) and
δ 50 ppm (13C). After acquiring the spectra, the NMR samples were evaporated to dryness under an N2 flow and subsequently stored in a freezer at −20°C.

2.7 qNMR

The total isolated amount was determined for each compound using the PULCON quantitative NMR method.[17–19] High purity benzoic acid (TraceCERT®, Sigma-Aldrich) was accurately weighed in and used as an external standard. The isolated amounts were expressed in μg dry weight. A rough estimate of the purities of the isolated compounds was determined by dividing the sum of integrals of the target compound by the sum of integrals of all signals (except the solvent signals) present in the 1H NMR spectrum.

3 RESULTS

3.1 Separation and clean up strategy

A schematic overview of the procedure for isolation of metabolites from urine is given in Figure 1. In short, after a first clean up and concentration by SPE, a urine concentrate was injected multiple times onto a preparative LC column, fractions containing conjugated valerolactones were identified using MS. The collected fractions were concentrated by SPE and further separated using an orthogonal analytical column where MS guides the separation of conjugated valerolactones. 1D 1H and 2D homo- and heteronuclear NMR experiments were used for the assignment of 1H and 13C NMR resonances of the isolated fractions. The PULCON principle was used for quantification. In the next sections, critical steps in the procedure are discussed in detail.

3.2 SPE clean up and prepLC–MS separation

First, 150 ml of urine was cleaned up and preconcentrated using SPE. Subsequently, the SPE eluent was evaporated and dissolved in 7.5 ml of 11% acetonitrile in milliQ water containing 0.1% (v/v) formic acid. Injection volumes of 500 μL were used. On the basis of data from the LC–MS spectrum and previously identified compounds,[12] we were able to detect five (suspected) conjugated valerolactones in the cleaned up, preconcentrated urine sample. Selection criteria were molecular mass of previously identified valerolactones[10,12] and loss of glucuronide and sulphate fragments. A typical PrepLC–MS chromatogram is given in Figure 2. For visual reference, the m/z values of these
compounds (Table 1) were extracted, and an overlay with the base peak chromatogram is presented. All target compounds were collected manually in individual glass containers.

3.3 | SPE of PrepLC–MS fractions

In total, five fractions were collected from one single run. Due to the high flow rate (3 ml/min) used for the PrepLC–MS method, the collected fraction volumes were large, which would result in lengthy drying times. Therefore, the collected fractions were cleaned up using SPE. Because the fractions contained up to 20% (v/v) acetonitrile, they had to be diluted to ensure that the compounds would absorb to the SPE cartridge material. The cartridges were eluted with methanol. After this SPE step, qualitative 1D ¹H NMR spectra were recorded to confirm the presence of the target compounds. From the 1D ¹H NMR spectra, it was concluded that the fractions still contained high levels of impurities and that the concentration of the target compounds was too low for structural elucidation. Therefore, it was necessary to repeat the fractionation step using the preparative LC column another 14 times.

3.4 | LC-MS fraction purification

The five fractions that were obtained using the PrepLC-MS method (15 injections of 500 μl urine) were of insufficient purity. To clean up these fractions, tailored orthogonal LC-MS methods need to be put in place for each fraction to isolate the compound of interest from other compounds. Scout runs were made with small injection volumes (2 μl), based on the observed separation between the retention times of the target compounds and impurities. For each compound, the eluent composition at the elution time from the PrepLC-MS method was calculated. This composition was used as start eluent composition for the second separation step. From here, a slow linear gradient (typically 1% over 90 min) was applied. Typically, two-three scout runs were required to obtain the final LC gradient. Here, the

| Compound name | Compound codes | Molecular formula | Elucidated structure |
|---------------|----------------|-------------------|---------------------|
| 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone-3′-O-glucuronide | UT0059 RUSMDDXSDLDDLKU-HPMKMSPSA-N | C₁₇H₁₉O₁₁ | ![Structure](image1) |
| 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-4′-O-glucuronide | UT0056 OTBIYBQMPICIK-KQKFAQMISA-N | C₁₇H₁₉O₁₀ | ![Structure](image2) |
| 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-3′-O-glucuronide | UT0055 UVGDTVGPBLMQLY-QKQFAQMISA-N | C₁₇H₁₉O₁₀ | ![Structure](image3) |
| 5-(3′-hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide | UT0065 DPSUQBKEHXATY-HVCLVNYSA-N | C₁₇H₁₉O₉ | ![Structure](image4) |
| 5-(3′-hydroxyphenyl)-γ-valerolactone-3′-O-sulphate | UT0067 DPRDYFJWDRNYAZ-SECBINFHSA-N | C₁₁H₁₁O₆S | ![Structure](image5) |
m/z values of the target compound (Table 1) were monitored using the mass spectrometer. For the second orthogonal LC-MS separation, the composition of the eluent was too polar for the metabolites in the trapped fractions to show affinity for the Waters HLB sorbent in the SPE. This hampered the use of an automatic LC-SPE system for trapping selected compounds, hence metabolites were obtained by collecting the fractions in an MS-guided manner. These fractions were subsequently dried under a gentle stream of nitrogen.

FIGURE 3 1D $^1$H NMR spectral regions containing the aromatic and glucuronide signals of isolated conjugated valerolactones, from top to bottom 5-(3',5'-dihydroxyphenyl)-γ-valerolactone-3'-O-glucuronide (UT0059), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone-4'-O-glucuronide (UT0056), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone-3'-O-glucuronide (UT0055), 5-(3'-hydroxyphenyl)-γ-valerolactone-3'-O-glucuronide (UT0065), and 5-(3'-hydroxyphenyl)-γ-valerolactone-3'-O-sulphate (UT0067)
3.5 | \( ^1\)H and \(^{13}\)C NMR spectral assignments

Figure 3 shows \(^1\)H NMR spectral regions of interest of the isolated valerolactones. The spectra show good signal-to-noise ratios, and only minor signals of impurities can be observed. This prompted us to record homo- and heteronuclear NMR spectra for full spectral assignment of \(^1\)H and \(^{13}\)C NMR resonances. For all fractions, 2D \(^1\)H-\(^1\)H TOCSY and \(^1\)H-\(^{13}\)C HSQC spectra could be obtained with sufficient signal-to-noise ratios. For fractions where more than 25 μg of material was isolated (UT0055 and UT0065), good quality 2D \(^1\)H-\(^{13}\)C HMBC spectra could be recorded. As an example, the complete assignment of the NMR signals (in both 1D and 2D spectra) for 5-(3′,4′-dihydroxyphenyl)-\(\gamma\)-valerolactone-3′-0-glucuronide (UT0055) is given in Figures 3, 4, and 5. J-coupling constants of around 6 Hz were observed between the H4 and H5 protons, indicating the valerolactones adopt an L-conformation. Using the combined information from the MS and 1D \(^1\)H NMR spectra and 2D TOCSY, HSQC, and HMBC NMR spectra, the structural elucidation for all five metabolites at confidence level MSI = 1\(^{[20]}\) could be confirmed. An overview of the identified compounds is given in Table 2. For each identified compound, the respective coupling constants, \(^1\)H chemical shifts and \(^{13}\)C chemical shifts (from HSQC experiments) are given in Table 3. These spectral assignments correspond well with those from spectral predictions on the basis of quantum mechanical principles.\(^{[21]}\) The mean error of the chemical shift prediction was 0.11 ppm, with a standard deviation of 0.09 ppm. The \(^1\)H spectral assignments as well as the corresponding quantum mechanical predictions have been deposited in the MetIDB database.\(^{[22]}\)

3.6 | Amount and purity quantification of the isolated compounds

For all five isolated compounds, quantitative 1D \(^1\)H NMR spectra were recorded, and the total isolated amount was determined using PULCON. The isolated amounts ranged between 8 and 160 μg, with a purity between 70% and 99%. For each compound, the isolated amount and the purity are given in Table 3.

4 | DISCUSSION

In this work, we demonstrated that by off-line hyphenation of SPE, prepLC, and analytical LC we can obtain preparative amounts of polyphenol metabolites from urine of volunteers that consumed their habitual amounts of tea. In the hands of an experienced operator, the current workflow for isolation of the conjugated metabolites from the urine matrix takes approximately 2 weeks. Even in its current form, the duration and effort of the presented workflow is modest compared with \(de novo\) synthesis. Furthermore, the workflow is amenable for automation. The throughput of the urine clean up and preconcentration SPE step can be enhanced by using SPE columns suitable for large sample volumes.
(more sorbent per cartridge) and implementation in a robotised setup. For the second orthogonal LC-MS separation step, no use could be made of an LC-SPE system for automatic (SPE) trapping of selected compounds using an \( m/z \) trigger or a predefined retention window. This calls for testing of other analytical LC columns with different separation characteristics, so compounds can be eluted using a lower percentage of organic solvent.

In some cases, compound instability at room temperature during long (20+ hr) NMR measurements times was observed. It is therefore recommended to isolate at least 25 µg of compound, to significantly reduce the measurement time of the 2D NMR experiments. Using our approach, we were able to isolate sufficient quantities for testing in \textit{in vitro} bioactivity assays\textsuperscript{[6]} as well as for use as analytical standards to prepare calibration curves for quantification in plasma by LC-MS.\textsuperscript{[18]}

**FIGURE 5** Aromatic (top) and aliphatic (bottom) regions of the 2D \( ^1\text{H} - ^1\text{H} \) TOCSY NMR spectrum of 5-(3',4'-dihydroxyphenyl)-\( \gamma \)-valerolactone-3'-O-glucuronide (UT0055) in methanol-\textit{d}_4 (300 K). Annotations correspond to assignments in the molecular structures.
TABLE 2 1H and 13C chemical shifts (ppm), signal multiplicity and 1H coupling constants (Hz) of the isolated compounds

| Compound | 1H | 13C |
|----------|----|-----|
| 5-(3',5'-dihydroxyphenyl)-γ-valerolactone-3'-O-glucuronide UT0059 | | |
| H3a      | 1.96 m | 28.7 |
| H3b      | 2.26 m |  |  |
| H2a      | 2.36 m | 30.2 |
| H2b      | 2.49 m |  |  |
| H4       | 4.76* m | 83.5 |
| H6a      | 2.84 dd 6.3, 14.0 | 42.9 |
| H5b      | 2.93 dd 6.3, 14.0 |  |  |
| H2'      | 6.50 br. s 1.6 | 111.0 |
| H3'      | 6.45 br. s 2.2 | 104.5 |
| H5'      | 7.05 d 8.3 | 119.3 |
| H6'      | 6.38 br. s 1.8 | 112.9 |
| H7       | 4.87* d 7.3 | 103.2 |
| H8       | 3.57 t 9.0 | 74.0 |
| H9       | 3.47 m | 75.3 |
| H10      | 3.87 d 9.8 | 77.3 |
| H11      | 3.95 d 9.8 |  |  |

*: From HSQC.
**: Overlaps with residual HDO signal.

5 | CONCLUSIONS

Preparative isolation of urinary metabolites of polyphenol metabolites from urine can be achieved by combining SPE for sample clean up, fractionation using a preparative LC column (coupled to MS) and subsequent purification of the fractions using an analytical LC column (coupled to MS). The clean up and preconcentration by SPE are critical for removing the high background levels of semipurified metabolites. The implementation of a preparative LC column instead of an analytical column significantly reduced the time required to isolate sufficient quantities of metabolite required for NMR analysis. The work yielded sufficient amounts of purified metabolites to allow for full spectral assignments of five conjugated valerolactones by means of 1D and 2D NMR. The use of
PULCON allowed for quantification of the isolated metabolites, of which the amounts ranged between 8 and 160 μg, with a purity between 70% and 91%. Such quantities allow the use the isolated metabolites as authentic standards for quantification by mass spectrometry and for testing of bioactivity in bioassays.

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CONFLICT OF INTEREST

The authors are employed by a company that markets tea products.

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