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ORIGINAL

# The Simultaneous Determination of Tricarboxylic Acid Cycle Acids and 2-Hydroxyglutarate in Serum from Patients with Nasopharyngeal Carcinoma Via GC–MS

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**Abstract** The tricarboxylic acid (TCA) cycle has recently been in the spotlight in the field of oncology. Mutations in the enzymes called cytosolic isocitrate dehydrogenases (IDHs) are a common feature in several types of cancers and provide the enzyme with the new ability to catalyze α-ketoglutarate (αKG) to 2-hydroxyglutarate (2HG), which is now considered to be a major contributor to the oncogenic activity of IDHs mutations. In this study, we describe the development and full validation of a gas chromatography–mass spectrometry (GC–MS) method for the simultaneous quantitative determination of serum TCA cycle metabolites and 2HG in nasopharyngeal carcinoma serum in the same workflow. All eight organic acids observed in the serum sample showed good linearity in the corresponding linear range during the same run. The linear ranges of the method were 10–680 ng $mL^{-1}$ for succinic acid, 10–640 ng $mL^{-1}$ for fumaric acid, 10–520 ng $mL^{-1}$ for malic acid, 40–860 ng $mL^{-1}$ for oxaloacetic acid, 36–780 ng $mL^{-1}$ for 2HG, 38–840 ng $mL^{-1}$ for αKG, 40–960 ng $mL^{-1}$ for aconitic acid and 68–6800 ng $mL^{-1}$ for citric acid. The recovery and reproducibility were satisfactory, and the data demonstrate that the method is simple, sensitive, and suitable for nasopharyngeal carcinoma serum analysis.

**Keywords** TCA cycle acids · 2HG · Nasopharyngeal carcinoma · GC–MS

**Abbreviations**

| | |
|---|---|
| CSF | Cerebrospinal fluid |
| CT | Computed tomography |
| GC–MS | Gas chromatography with mass spectrometry |
| 2HG | 2-Hydroxyglutarate |
| IDH1 | Isocitrate dehydrogenase 1 |
| αKG | α-Ketoglutarate |
| LLOD | Lower limit of detection |
| LLOQ | Lower limit of quantification |
| LC-MS | Liquid chromatography-mass spectrometry |
| NPC | Nasopharyngeal carcinoma |
| MRI | Magnetic resonance imaging |
| SIM | Selective ion monitoring |
| S/N | Signal-to-noise ratio |
| TCA | Tricarboxylic acid cycle |

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## Introduction

The tricarboxylic acid (TCA) cycle is the eventual metabolic pathway of the three major nutrients (sugar, amino acids and lipid), and it is the most important metabolic pathway for energy production in the body. In the TCA cycle, citric acid, aconitic acid, αKG, fumaric acid, malic acid, succinic acid and oxaloacetic acid are successively involved. The mutations of IDH1 and IDH2 directly convert αKG to 2HG [1, 2]. TCA cycle metabolites and 2HG metabolic disorders are related to many types of cancers in humans, such as primary human brain cancers, acute myeloid leukemia and peripheral T cell lymphoma [3–5]. Therefore, the simultaneous determination of TCA cycle metabolites and 2HG may be important in the pathogenesis, diagnosis, and monitoring of such disease conditions.

Nasopharyngeal carcinoma (NPC), a prevalent cancer in Southeast China, originates in the nasopharyngeal mucosal epithelium and gland [6]. A few studies on NPC that are associated with serum metabolites have been reported [7]. Thus, the determination of the contents of the TCA cycle metabolites and 2HG in the serum of NPC patients provides the basis for the pathogenesis of NPC in the metabolic pathway. To date, many methods have been developed to analyze the TCA cycle metabolites and 2HG in biological fluids, cancer cells and tumor tissues [8–22]. However, most of them measure either TCA cycle metabolites or 2HG [8–21]. A few methods can simultaneously determine TCA metabolites and 2HG in a single run, but only a few types of TCA cycle metabolites were determined [22].

This study develops a simple method for the simultaneous and rapid determination of TCA cycle metabolites and 2HG in a single run with GC–MS. We apply the method to determine serum TCA cycle metabolites and 2HG levels of NPC patients to explore the clinical significances of these metabolites for NPC.

## Materials and Methods

### Chemicals and Reagents

Citric acid, αKG, 2HG, aconitic acid, fumaric acid, malic acid, succinic acid, oxalacetic acid, 2-isopropylmalic acid, methanol and N,O-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA + TMCS) were all purchased from Sigma-Aldrich (St. Louis, MO, USA) as analytical grade. The ultrapure water was obtained from a Millipore pure water purification system (Millipore-Q, Billerica, MA, USA). The stock solutions of succinic acid (170.0 μg $mL^{-1}$), fumaric acid (160.0 μg $mL^{-1}$), malic acid (130.0 μg $mL^{-1}$), oxalacetic acid (200.0 μg $mL^{-1}$), 2HG (120.0 μg $mL^{-1}$), αKG (160.0 μg $mL^{-1}$), aconitic acid (120.0 μg $mL^{-1}$) and citric acid (170.0 μg $mL^{-1}$) were separately prepared in methanol. Stock solutions of these analytes were then mixed and diluted with methanol to provide seven standards of the desired concentration. We used 2-isopropylmalic acid as the internal standard (IS), and the IS solution was prepared in methanol to a concentration of 1.0 mg $mL^{-1}$. All of the solutions were stored in the dark at 4 °C in a refrigerator.

### Instruments

The GC–MS system consists of a 7890 GC system (Agilent Technologies, CA, USA), a 5975 inert XL MSD with a triple-axis detector (Agilent Technologies, CA, USA), a 7693 autosampler (Agilent Technologies, CA, USA) and a 5 % phenyl methyl siloxane analytical column (30 m × 0.25 mm i.d.; film thickness: 25 μm) (19091J.433, HP; Agilent Technologies, CA, USA). The pressure blowing concentrator was obtained from HGC-24, Heng'ao Company in Tianjin, China.

### Sample Collection

Thirty-three healthy control subjects (19 males and 14 females, 20–52 years of age at an average age of 43.5 years) and 32 nasopharyngeal carcinoma (NPC) patients (21 males and 11 females; 27–63 years of age with an average age of 45.1 years) were obtained from the Xiangya Hospital at Central South University. NPC was defined by a routine diagnostic workup that included a detailed clinical examination of the head and neck, nasopharyngoscopy, histological and cytological examination of the tumor tissue, and radiological imaging techniques [computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonography]. The healthy control subjects were free from any physical illness for at least 2 weeks before the study. All subjects were provided with written informed consent upon entry into this study, and the Ethics Committee of the Xiangya Hospital of Central South University approved the protocol. Venous blood was collected separately from each NPC patient and from the healthy control subjects following an overnight fasting. The blood samples were centrifuged at 4000*g* for 10 min at room temperature, and then aliquots of sera were stored at −80 °C until analyzed.

### Sample Preparation

Approximately 100 μL of serum sample was mixed with 400 μL of a 95 % methanol aqueous solution (v/v), and then 10 μL of the IS solution (1.0 mg $mL^{-1}$) was added. The mixture was mixed via vigorous vortexing

for 1 min; then, it was centrifuged for 10 min at 16,000*g* at 4 °C. Approximately 430 μL of the resultant supernatant was transferred to a glass test tube (5 mL), and dried via nitrogen concentration at room temperature. Approximately 100 μL of bis-(trimethylsilyl) trifluoro acetamide (BSTFA + TMCS = 99:1) was added for the derivatization step, and the mixture was placed in a water bath for 1 h at 60 °C. The supernatant was used for the GC–MS measurements.

## GC–MS Data Acquisition

The derivatized samples (1 μL) were injected at a split ratio of 10:1 into an Agilent 7890A GC system coupled to an Agilent 5975C system GC/MSD (Agilent Technologies, CA, USA). A HP-5 MS quartz capillary column that was coated with 5 % phenyl methyl siloxane (30 m × 250 μm i.d., 0.25 μm film thickness; Agilent Technologies, CA, USA) was used to separate the derivatives. The initial temperature of the oven was held at 100 °C for 3 min, ramped to 240 °C at a rate of 11 °C $min^{-1}$, then ramped to 300 °C at a rate of 30 °C $min^{-1}$, and then held at 300 °C for 6 min. Helium was used as the carrier gas at a constant flow rate of 1 mL $min^{-1}$. The temperatures of the ion source and injector were 200 and 280 °C, respectively. The electron energy was 70 eV, and data were collected in the selective ion monitoring (SIM) mode (*m/z* 35–800) with a scan time of 0.2 s. The detector voltage was set to 0.9 kV, and the solvent delay and data acquisition times were 6.5 and 7.0 min, respectively.

In the SIM mode, the peaks of succinic acid, fumaric acid, malic acid, oxalacetic acid, 2HG, αKG, aconitic acid, citric acid and the IS in the serum were identified by matching the retention time and their abundant ions. The ions used for quantification of the metabolite levels are as follows: *m/z* 247 for succinic acid, *m/z* 245 for fumaric acid, *m/z* 335 for malic acid, *m/z* 333 for oxalacetic acid, *m/z* 347 for αKG, *m/z* 349 for 2HG, *m/z* 375 for aconitic acid, *m/z* 465 for citric acid, and *m/z* 275 for the IS.

## Method Validation

### *Chromatographic and Mass Conditions*

We used the GC system equipped with a HP-5 MS quartz capillary column to separate the analytes and the IS. To optimize the heating rate of the oven temperature and the flow rate of the carrier gas, the heating rate was varied from 5 to 12 °C $min^{-1}$, and the flow rate was varied from 0.8 to 1.2 mL $min^{-1}$. The SIM of the MS was used to detect the metabolites. The parameters for the electron energy and the detector voltage were further optimized to obtain the richest relative abundance of the characteristic ions for these metabolites.

### *Calibration Curves and Detection Limits*

Approximately 100 μL of the dilute mixed standard solution at seven desired amounts was transferred to 5 mL glass bottles that were all spiked with 10 μL of the IS solution. They were then dried and derived as described above, and the supernatants were determined in triplicate using the described GC-MS/SIM conditions. The calibration curves were established by plotting the ratio of the quantified ion peak area of these metabolites to the internal standard versus their respective amounts in the calibration samples. The regression parameters of the slope and correlation coefficient were calculated via the linear least square regression. According to the FAD guidelines, the lower limit of detection (LLOD) is defined as the lowest quantities of each analyte that can be reliably differentiated from background noise, as assessed with a blank sample. The lower limit of quantification (LLOQ) is defined as the lowest concentration on the calibration curve with an inter-day coefficient of variation (CV) of <20 % and an accuracy between 80 and 120 %. Because these analytes were present in the serum samples and obtaining a matrix blank was not possible, 100 μL of methanol was used as the blank sample to develop the standard curve.

### *Precision Evaluation*

Pooled serum samples that were spiked with each analyte at three levels were used to determine the intra- and inter-day precisions. The intra-day precision was assessed by performing six independent analyses of these samples during 1 day, whereas the inter-day precision was obtained by analyzing these samples every day over six consecutive days.

### *Recovery Experiments*

To estimate the recovery of the method, the pooled serum was divided into four groups. Group 1 was added to 0.4 mL methanol as the base sample, and groups 2–4 were added separately to 0.4 mL mixed standard solutions of methanol to produce high, middle and low concentrations. Each sample was tested in six replicates and calculated for their average recovery. Recovery was expressed as the [(found concentration − basic concentration)/spiked concentration] × 100 %.

### *Qualitative and Quantitative Analysis*

The presence of succinic acid, fumaric acid, malic acid, oxalacetic acid, 2HG, αKG, aconitic acid and citric acid in the serum was determined by comparing their peak retention times and characteristic peaks with those

of the external standards. Their amounts in the serum were determined from ratios of their quantification ion peak areas to the IS, each according to their standard equations.

*Application in Serum of Nasopharyngeal Carcinoma Patients*

Samples from 33 healthy control subjects and 32 NPC patients were collected and analyzed. This was performed to determine if there were significant different levels of succinic acid, fumaric acid, malic acid, oxalacetic acid, 2HG, αKG, aconitic acid and citric acid between the NPC and healthy control samples.

### Statistical Analysis

The data are shown as mean ± SD. The data between groups were evaluated statistically using Student's *t* test with SPSS 17.0 statistical software. A $p < 0.05$ was considered to be statistically significant.

## Results

### Chromatographic and Mass Conditions

After examination of a range of chromatographic conditions the separation shown in Fig. 1, and described in detail in the experimental, was selected for validation. The eight organic acids and the IS were separated well (resolution >1.5) under these conditions. The retention time and quantification ion (*m/z*) are shown in Table 1.

### Linearity and Detection Limit

Each calibration curve was prepared in methanol with seven different amounts of chemical markers. The linear equations, correlation coefficients ($r^2$), linearity ranges, and the lower limits of detection (LLOD) and quantification (LLOQ) for the target metabolites are shown in Table 1. The *X* indicates the quantities of these metabolites. The *Y* indicates the ratio of quantification ion peak area of the analyte to the IS. The correlation coefficients ($r^2$) were all greater than 0.9978, showing good linearity in their respective linear ranges for these metabolites. The lower limits of detection (LLOD) for succinic acid, fumaric acid, malic acid, oxalacetic acid, 2HG, αKG, aconitic acid and citric acid were 2, 2, 2, 12, 10, 12, 12 and 20 ng $mL^{-1}$, respectively, exhibiting the high sensitivity for determining the levels of these metabolites in serum of NPC patients.

### Precision

The precision data are shown in Table 2. For all concentrations, the RSD was found to be less than 12.7 %. The values were acceptable for analysis of biological samples, demonstrating that a reliable and reproducible method was established for the determination of these metabolites in serum. The results also showed that the TMS derivatives of these metabolites were stable for 6 days.

### Recoveries

The mean recovery percentages of the eight analytes from serum were all greater than 81.1 % at three concentration levels (Table 2), indicating that the recoveries of the analytes were consistent, precise and reproducible at different concentration levels in serum.

### Application for Serum of Nasopharyngeal Carcinoma Patients

The present method was successfully used to analyze target metabolites in NPC and healthy control subjects. Representative mas chromatograms of serum samples from a healthy control and a NPC patient are shown in Fig. 1. As shown in Table 3, compared with healthy control subjects, the serum quantities of citric acid and aconitic acid in the NPC patients increased, and there were significant differences between the NPC patients and the healthy control subjects ($p < 0.05$). The concentration of malic acid significantly decreased compared to the healthy control subjects ($p < 0.05$), whereas the amounts of oxalacetic acid, αKG, succinic acid fumaric acid and 2HG showed no significant changes ($p > 0.05$).

## Discussion

An increasing number of studies focus on the detection of TCA cycle metabolites and 2HG using mass spectrometry. Dang et al. [1] used liquid chromatography-electrospray ionization-mass spectrometry (LC–MS) with multiple reaction-monitoring (MRM) modes to determine the levels of 2HG and TCA metabolites in cell and culture media. However, 2HG and TCA metabolites were determined separately. Additionally, Balss et al. [3] used reversed–phase liquid chromatography coupled with mass spectrometry (LC–MS) to determine serum 2HG in acute myeloid leukemia; however, they did not determine TCA cycle metabolites in serum. Moreover, Calderon-Santiago et al. [10] used gas chromatography couped with mass spectrometry with selective ion mode (GC–MS/SIM) to measure TCA

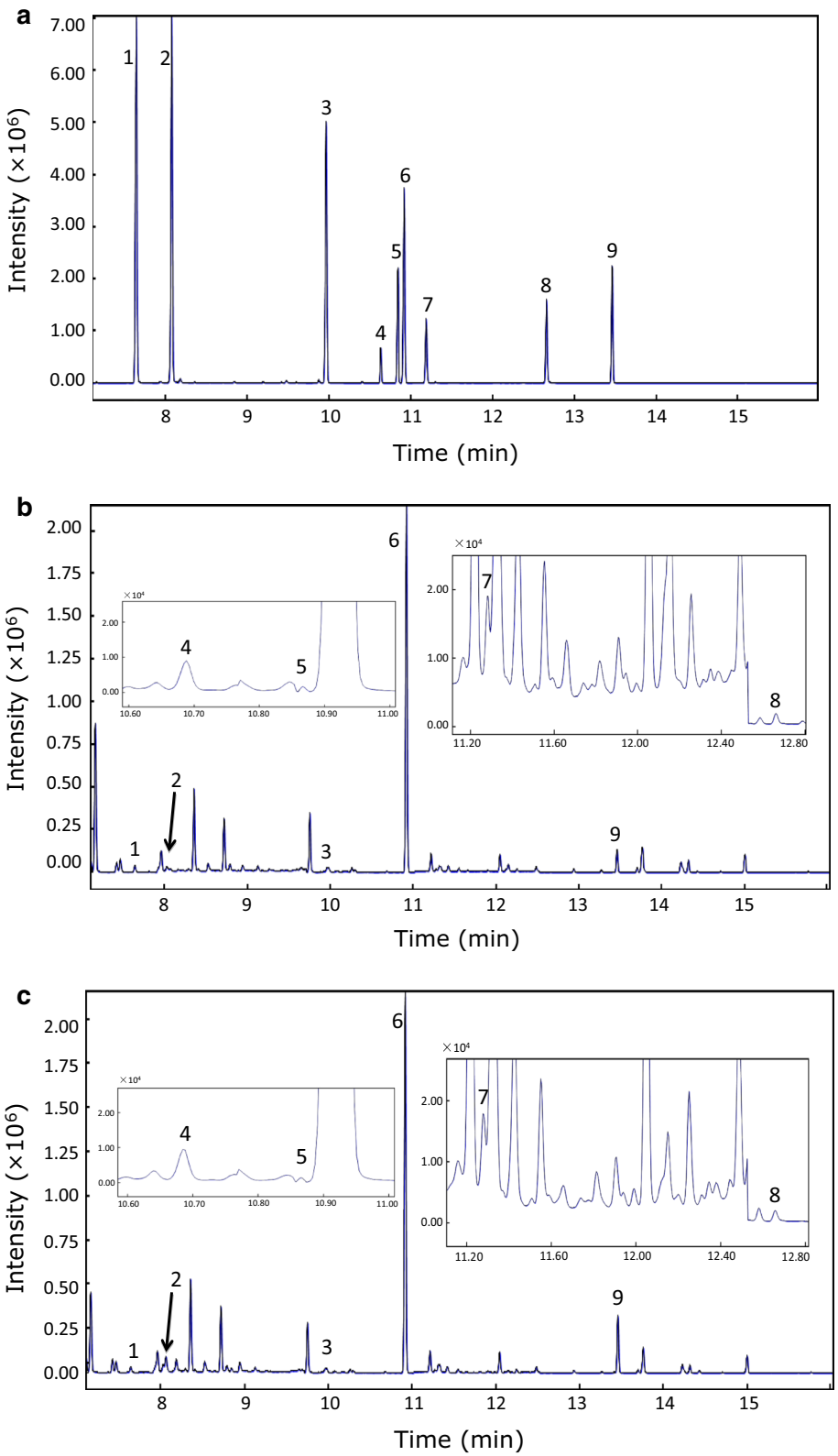


**Fig. 1** Selective ion mode chromatograms from a mixed standard sample containing 5 μg $mL^{-1}$ of target metabolites and (**a**), a serum sample from a healthy control (**b**) and a serum sample from a patient with NPC (**c**), all spiked with the internal standard (IS). *1* Succinic acid, *2* fumaric acid, *3* malic acid, *4* oxalacetic acid, *5* 2HG, *6* 2-isopropylmalic acid (IS), *7* αKG, *8* aconitic acid, *9* citric acid

cycle metabolites in human serum samples from patients with cardiovascular disorders, but they did not determine 2HG. Koivunen et al. [22] ever reported simultaneous determination of 2HG, αKG and succinic acid by LC–MS in negative mode using multiple reaction monitoring (MRM) on a Quattro micro triple quadruple mass

**Table 1** The retention time, quantification ion, regression equations and detection limits for the eight analytes

| Analytes | Retention time (min) | Quantification ion (*m/z*) | Regression equation[a] | $r^2$ | Linear range (ng mL$^{-1}$) | LLOD (ng mL$^{-1}$) | LLOQ (ng mL$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Succinic acid | 7.642 | 247 | $Y = 0.2793X - 0.0008$ | 0.9997 | 10–680 | 2 | 10 |
| Fumaric acid | 8.078 | 245 | $Y = 0.1630X - 0.0052$ | 0.9999 | 10–640 | 2 | 10 |
| Malic acid | 9.959 | 335 | $Y = 0.1356X - 0.002$ | 0.9989 | 10–520 | 2 | 10 |
| Oxalacetic acid | 10.686 | 333 | $Y = 0.0013X + 6E - 05$ | 0.9978 | 40–860 | 12 | 40 |
| 2HG | 10.908 | 349 | $Y = 0.0272X + 0.0002$ | 0.9993 | 36–780 | 10 | 36 |
| α-KG | 11.267 | 347 | $Y = 0.0039X - 0.0008$ | 0.9995 | 38–840 | 11 | 38 |
| Aconitic acid | 12.737 | 375 | $Y = 0.0222X - 0.0017$ | 0.9987 | 40–960 | 12 | 40 |
| Citric acid | 13.524 | 465 | $Y = 0.5954X - 0.0509$ | 0.9992 | 68–6800 | 20 | 68 |
| IS | 10.917 | 275 | | | | | |

[a] In the linear regression equation, $X$ is expressed as the analyte concentration (μg mL$^{-1}$) and $Y$ is expressed as the quantification ion peak area of the analyte to the IS

$r^2$ correlation coefficient of determination of the linear regression equation

**Table 2** Precision and recovery of the eight analytes in serum ($n = 6$)

| Analytes | Concentration (spiked) (ng mL$^{-1}$) | Intra-day RSD (%) | Inter-day RSD (%) | Recovery (%, mean ± SD) |
|---|---|---|---|---|
| Succinic acid | 109.1[a] (42.5)[b] | 5.7 | 6.7 | 105.1 ± 6.9 |
| | 109.1 (85) | 4.3 | 5.8 | 93.3 ± 5.1 |
| | 109.1 (170) | 5.9 | 7.2 | 97.6 ± 8.0 |
| Fumaric acid | 211.0 (40) | 6.4 | 7.6 | 103.9 ± 6.3 |
| | 211.0 (80) | 4.4 | 5.4 | 92.9 ± 5.1 |
| | 211.0 (160) | 7.2 | 7.3 | 96.2 ± 8.2 |
| Malic acid | 236.3 (65) | 8.7 | 9.1 | 91.2 ± 10.1 |
| | 236.3 (130) | 5.4 | 7.9 | 101.1 ± 9.6 |
| | 236.3 (260) | 7.8 | 10.3 | 93.3 ± 10.8 |
| Oxalacetic acid | 173.7 (50) | 10.0 | 12.7 | 81.1 ± 14.2 |
| | 173.7 (100) | 8.0 | 11.3 | 85.2 ± 11.7 |
| | 173.7 (200) | 9.2 | 11.9 | 90.5 ± 10.5 |
| 2HG | 68.4 (30) | 7.4 | 8.5 | 91.8 ± 7.2 |
| | 68.4 (60) | 5.6 | 6.7 | 89.6 ± 5.0 |
| | 68.4 (120) | 6.9 | 9.0 | 92.8 ± 8.3 |
| α-KG | 142.6 (30) | 8.5 | 9.8 | 90.9 ± 10.2 |
| | 142.6 (60) | 6.3 | 8.2 | 93.7 ± 7.0 |
| | 142.6 (120) | 6.7 | 7.3 | 102.5 ± 6.1 |
| Aconitic acid | 165.9 (30) | 8.2 | 9.5 | 89.4 ± 9.2 |
| | 165.9 (60) | 6.4 | 7.8 | 92.1 ± 5.4 |
| | 165.9 (120) | 7.9 | 9.1 | 90.3 ± 10.3 |
| Citric acid | 367.8 (100) | 9.4 | 10.1 | 85.7 ± 11.9 |
| | 367.8 (200) | 6.8 | 8.3 | 91.5 ± 10.3 |
| | 367.8 (400) | 7.7 | 9.2 | 87.0 ± 8.8 |
| IS | 1000.0[c] | 5.2 | 6.9 | 95.6 ± 5.7 |

[a] Concentration of each analyte in the pooled serum (ng mL$^{-1}$)

[b] Concentration of each analyte spiked in the pooled serum (ng mL$^{-1}$)

[c] Concentration of the IS spiked in the pooled serum (ng mL$^{-1}$)

**Table 3** Levels of the eight analytes in serum for the healthy control group and the NPC group ($\mu g\ mL^{-1}$, $\bar{x} \pm s$)

| Analytes | Control group ($n = 33$) | NPC group ($n = 32$) | $p$ value |
|---|---|---|---|
| Succinic acid | $0.137 \pm 0.049$ | $0.108 \pm 0.034$ | 0.108 |
| Fumaric acid | $0.226 \pm 0.096$ | $0.201 \pm 0.083$ | 0.492 |
| Malic acid | $0.234 \pm 0.081$ | $0.129 \pm 0.036$ | 0.001 |
| Oxalacetic acid | $0.174 \pm 0.080$ | $0.197 \pm 0.108$ | 0.143 |
| 2HG | $0.067 \pm 0.023$ | $0.055 \pm 0.024$ | 0.192 |
| α-KG | $0.138 \pm 0.064$ | $0.171 \pm 0.097$ | 0.161 |
| Aconitic acid | $0.152 \pm 0.043$ | $0.366 \pm 0.095$ | 0.005 |
| Citric acid | $0.362 \pm 0.176$ | $2.384 \pm 0.922$ | 0.00013 |

$\bar{x}$ mean values, $s$ standard deviations

spectrometer, but fumaric acid, malic acid, oxalacetic acid, aconitic acid and citric acid were not measured.

Here, we presented a validated GC–MS/SIM assay for the simultaneous determination of TCA cycle metabolites and 2HG in a single analytical run that is reliable, precise and reproducible. Based on FDA regulatory guidelines, this method is suitable to be applied in biological sample analysis. The assay achieved good analytical separation of all eight organic acids and IS with acceptable intraday and interday precisions and recoveries. This method had the ability to measure the eight analytes over a wide analytical range, and the linear range for succinic acid, fumaric acid, malic acid, αKG, aconitic acid and citric acid broadly covered the reference values reported previously by Calderon-Santiago et al. [10].

The present method was successfully used to analyze target metabolites in NPC and healthy control subjects. TCA cycle was found to be disturbed in NPC patients. The serum concentration of citric acid was increased in NPC patients, which coincides with the report from Tang et al. [7]. The evaluated citric acid can be exported to the cytosol and used for protein acetylation or fatty acid biosynthesis, which can contribute to the rapid proliferation of cancer cells [23]. Mutations of both IDH1and IDH2 have been found in various human cancers, including acute myeloid leukemia [4], colon cancer [24], osteosarcoma [25], prostate cancer [26], glioblastoma [27] and intrahepatic cholangiocarcinoma [28]. Oncogenic mutations of IDH1 and IDH2 can convert αKG to 2HG, which accumulates up to millimolar leverls in cancer cells and serum [1, 2, 16, 28]. The level of 2HG in NPC serum was not found to increase, providing there were no mutations in IDH1 and IDH2 in NPC.

## Conclusions

A novel GC–MS/SIM method for the simultaneous analysis of serum TCA cycle metabolites and 2HG was developed. In this method, all target metabolites were separated easily in one single run, and the run time was less than 24 min. The method was applied in the serum of NPC patients. Changes in the target metabolite contents suggested that there was likely a metabolic reprogramming in the TCA cycle and no mutations in IDH1 and IDH2 in the NPC patients. Our results provide new clues for the study of NPC, but the specific mechanism requires further study.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflicts of interest. This manuscript has been read and approved by all of the authors, and it has not been submitted and is not under consideration for publication elsewhere.

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