



A chemical derivatization based UHPLC-LTQ-Orbitrap mass spectrometry method for accurate quantification of short-chain fatty acids in bronchoalveolar lavage fluid of asthma mice

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ABSTRACT

Recent studies have demonstrated the important role of short-chain fatty acids (SCFAs) in the maintenance of homeostasis of respiratory immunity. However, there is still no report focus on the determination of SCFAs level in bronchoalveolar lavage fluid (BALF), the most common sample used for screening biomarkers of the pulmonary diseases. Herein, an ultra-high-performance liquid chromatography with LTQ-Orbitrap mass spectrometer (UHPLC-LTQ-Orbitrap) oriented 3-nitrophenylhydrazine (3-NPH)-based derivatization method was developed for the quantification of SCFAs in BALF. To achieve accurate quantitation, d4-acetate was used as internal standard to compensate for the matrix effects. Method validation showed a good linearity ($R^2 > 0.9992$) with wide concentration range, and the intra-day and inter-day precision for determination of eight SCFAs in BALF samples was $\leq 14.79\%$. The quantitation accuracy, assessed by relative recoveries, ranged from 90% to 110% for target SCFAs at three concentration levels. Matrix effects ranged from 85% to 115%, and the lower limits of quantification of these targeted SCFAs were varied from 3 to 24 nmol/L. The SCFAs-targeted method was then applied to determine the changed levels in BALF samples from OVA-induced asthma mice and normal mice. In addition, the universality of our developed method was also demonstrated by determining the SCFAs concentrations in feces, serum and lung tissue samples from asthma and normal mice. These results indicate that 3-NPH derivatization based UHPLC-LTQ-Orbitrap provides accurate view of global SCFAs alternation in different samples, giving a support to deduce the origin of SCFAs in lung. The present study is of great importance for understanding the role of SCFAs in modulation of host metabolism and immunity.

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1. Introduction

Short-chain fatty acids (SCFAs) are a subset of fatty acids produced by the gut microbiota during the fermentation of fibers and dietary carbohydrates [1]. There is now a growing evidence to show

that SCFAs play an important role in the maintenance of health and the development of disease [2]. Some of these studies even suggested a role for the gut microbiota-derived SCFAs in modulating respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [3–5]. Interestingly, independent investigations have recently demonstrated that the human respiratory tract (spans from the nostrils to the lung alveoli) is also inhabited by niche-specific communities of bacteria [6]. Therefore, it is intriguing that the respiratory microbiota-derived SCFAs might also be involved in the modulation of host metabolism and immunity.

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However, rare research has focused on the determination of respiratory microbiota-derived SCFAs, which limits the understanding of their possible functions in maintenance of homeostasis of respiratory immunity.

Bronchoalveolar lavage (BAL) is often used in immunological researches as a mean of sampling cells or pathogen levels in the lung [7]. And bronchoalveolar lavage fluid (BALF) is the most common sample used to determine inflammatory cells, cytokines, and protein composition of the pulmonary airways [8–11]. Recently, BALF samples are being investigated to screen metabolite biomarkers of lung disease. Analytical platforms for metabolite profiling of BALF include high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC/QTOF-MS)-based lipidomics and metabolomics, and gas chromatography-mass spectrometry (GC/MS)-based metabolomics [12–15]. The monitors of metabolome in BALF could reveal comprehensive pulmonary metabolic signatures in lung disease through specific alterations in carbohydrates, lipids, sterols, and energy metabolic pathways [14,16]. However, up to now, no method has been reported for the determination of SCFAs in BALF samples. Its utility as a biofluid for SCFAs quantitation may be limited because of its high protein and salt content but low concentrations of SCFAs [17].

Fortunately, several analytical techniques which mainly included chemical derivatization procedure coupled with GC/MS or LC-MS have been used to analyze the SCFAs in other biological samples, such as blood, urine and feces samples [18–22]. Based on the previous studies, we intend to use 3-nitrophenylhydrazine (3-NPH) to derivatize SCFAs in BALF for ultra-high-performance liquid chromatography with LTQ-Orbitrap mass spectrometer (UHPLC-LTQ-Orbitrap) quantitative analysis. Because of the high speed of MS scans and the high efficiency of high energy collision-induced dissociation on the LTQ-Orbitrap instrument, the MS/MS spectra can be readily acquired for the derivatized SCFAs [23,24]. Its high-ion-transmission results in short accumulation times, which distinguishes it for the purpose of LC-MS analysis [24–26]. This merit appears attractive for overcoming the main problems encountered with quadrupole instruments, especially for their low mass resolution resulted significant overlapping problems in the analysis of complex metabolite mixtures [24,27]. Herein, 3-NPH was used for pre-analytical chemical derivatization by quantitatively converting BALF-derived SCFAs to their 3-nitrophenylhydrazones under a mild reaction condition for subsequent UHPLC-LTQ-Orbitrap analysis. To the best of our knowledge, this is the first report about quantitative analysis of SCFAs in BALF. The profile of SCFAs in BALF has been successfully applied to discriminate between asthmatic mice and normal controls.

2. Materials and methods

2.1. Chemicals and reagents

Standards: acetate (C_2 , 99.8%), propionate (C_3 , 99.5%), isobutyrate (C_4 , 99.5%), butyrate (C_4 , 99.5%) and valerate (C_5 , 99.5%) were purchased from Shanghai Macklin Biochemical Co. Ltd (Shanghai, China). Isovalerate (C_5 , 99.5%), 2-methylbutyrate (C_5 , 98%), caproate (C_6 , 99.5%) and d4-acetate (99.5%) were purchased from Aladdin (Shanghai, China). Derivatization reagents: 3-nitrophenylhydrazine hydrochloride (3-NPH) and 1-ethyl (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl) were purchased from Aladdin (Shanghai, China); pyridine was purchased from Sinopharm Chemical Reagent Co. Ltd, R.P.C. (Shanghai, China). LC-MS grade acetonitrile (ACN) and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by filtration of distilled water using a Milli-Q system (Millipore, USA).

2.2. Sampling bronchoalveolar lavage fluid from asthmatic mice

Female BALB/c mice (18–20 g, 6–8 weeks old) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). All animals were maintained in isolation under controlled light and temperature conditions. BALB/c mice were subcutaneously (s.c.) sensitized to ovalbumin (OVA, 50 μ g; Grade BR, yuanye Bio-Techology Co., Ltd, Shanghai, China) with 50 μ L of Incomplete Freund's Adjuvant (IFA; Sigma, U.S.A.) in 50 μ L of 0.9% saline on days 0, 7 and 14. On day 22–28, mice were challenged with an aerosolized solution of 5% OVA (w/v) in 0.9% saline for 30 min every day. The mice of control group were treated with saline instead of the OVA/IFA solution during immunization and challenge. Bronchoalveolar lavage was performed by using a cannula surgically inserted into the trachea. Ice-cold PBS (1.0 mL $\times$ 3) was instilled into the airways and a consistent volume of BALF (2.4–3.0 mL) was recovered from each mouse. BALF samples were kept at -80°C prior further analysis.

2.3. The preparation of BALF samples and 3-NPH-based derivatization

After lyophilization [28], each BALF sample was redissolved in 90 μ L acetonitrile. The solution was then vortexed for 2 min followed by centrifugation at 12,000 rpm for 10 min. And then, 20 μ L of the supernatant was transferred to an eppendorf tube for further derivatization.

The derivative reagent (2 mL) was constituted of 1 mL of 200 mmol/L 3-NPH in 50% acetonitrile, 200 μ L of 600 mmol/L EDC in 50% acetonitrile, and 800 μ L 7.5% pyridine in 50% acetonitrile. The samples were mixed with derivative reagent at the ratio of 1:1 (v/v; 40 μ L in total). The whole process was performed in an ice bath. Afterwards, the mixture was permitted to react in a water bath at 40°C for 30 min. After reaction, the mixture was cooled down at 4°C and finally diluted to 1 mL with 10% acetonitrile. Before injection, the mixed system was centrifuged at 12,000 rpm for 10 min, and 720 μ L of the supernatant was added with 30 μ L of 3-NPH-derivatized d4-acetate (internal standard, IS) at a final concentration of 800 nmol/L.

2.4. Preparation of standard solutions, calibration and quality control (QC) samples

The mixture of stock standard solution containing acetate (10 mmol/L), propionate (5 mmol/L), isobutyrate (2.5 mmol/L), butyrate (5 mmol/L), 2-methylbutyrate (1.25 mmol/L), isovalerate (1.25 mmol/L), valerate (2.5 mmol/L) and caproate (10 mmol/L) was prepared in 50% acetonitrile. Before use, the mixture was diluted with 50% acetonitrile to give a series of mixed standards working solutions. The d4-acetate stock solution (1 mmol/L) was also prepared in 50% acetonitrile, which was reacted as above process to prepare 3-NPH-derivatized d4-acetate (IS, 20 μ mol/L). Quality control (QC) samples were prepared from each biological sample to ensure broad metabolite coverage. All solutions were stored at 4°C before use.

2.5. The solvent-only and matrix-matched calibration curves

To prepare the solvent-only curve, 90 μ L of acetonitrile was spiked with 10 μ L mixed standards working solution series, respectively. After being homogenized, 20 μ L of the mixed solution was transferred for derivatization, and was diluted to 1.0 mL with 10% acetonitrile followed by centrifugation. Then, 30 μ L of IS working solution (1 mmol/L) was added to 720 μ L of sample supernatant to prepare working solution series (96–15360 nmol/L for acetate, 9.6–7680 nmol/L for propionate, 96–3840 nmol/L for

isobutyrate, 0.096–7680 nmol/L for butyrate, 2.4–1920 nmol/L for 2-methylbutyrate, 2.4–1920 nmol/L for isovalerate, 0.048–3840 nmol/L for valerate, 96–15360 nmol/L for caproate and 800 nmol/L for IS). Before making matrix-matched calibration curve, 90 μ L of QC sample was spiked with 10 μ L mixed standards working solution series, respectively. The following procedures were the same as that for the solvent-only curve. At last, the curve concentrations were 24–19200 nmol/L for acetate, 12–9600 nmol/L for propionate, 6–4800 nmol/L for isobutyrate, 12–9600 nmol/L for butyrate, 3–2400 nmol/L for 2-methylbutyrate, 3–2400 nmol/L for isovalerate, 6–4800 nmol/L for valerate, 96–19200 nmol/L for caproate and 800 nmol/L for IS.

2.6. UHPLC-MS/MS conditions

UHPLC-MS/MS analysis was performed using a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) and a LTQ ORBITRAP VELOSPRO mass spectrometer equipped with heated electrospray ionization (HESI) prob (Thermo Fisher Scientific, San Jose, CA, USA).

Reversed-phase chromatography was used to chromatographic separation on a Waters Acquity BEH C₁₈ column (2.1 $\times$ 100 mm, 1.7 μ m). The mobile phases were composed of 0.01% formic acid in water (v/v) (A) and 0.01% formic acid in acetonitrile (v/v) (B). The mobile phases were eluted at 0.35 mL/min with the gradient as follows: 0–2 min 15% B; 2–11 min 15–55% B; 11–12 min 55–100% B; 12–13 min 100% B; 13–13.5 min 100%–15%, re-equilibrate at 15% B for 5 min. The column temperature was maintained at 40 °C, and the injection volume was 2 μ L.

The MS/MS detection was performed via electrospray ionization source in the negative mode. The parameters of mass spectrometer included a transfer tube temperature of 350 °C; a sheath gas of 35 arb; an auxiliary gas of 10 arb; a sweep gas of 0 arb; an ion spray voltage of 3.0 kV; a collision energy of 30 eV and a capillary temperature of 275 °C. Selective Reaction Monitor (SRM) conditions for the quantification of each compound were summarized in Table 1.

The quantitation of SCFAs in BALF was carried out using Xcalibur Quan Browser (Thermo Finnigan, U.S.A). Calibration curves for the targeted SCFAs were constructed by plotting each SCFA/IS peak area ratios obtained from calibration standards versus amounts of SCFA and fitting these data using linear regression with the correlation coefficient (R^2).

2.7. Method validation

The quantitative study requires to be simple, reliable, and comprehensive. To meet these requirements, the developed method was validated with respect to the linearity range, repeatability, precision, recovery, derivatization reaction yield and matrix effect.

The linearity was determined from the calibration curves constructed for each analyte in solvent blanks and QC samples, respectively. Linearity was acceptable if R^2 was ≥ 0.999 . The intra-assay variability of the method was determined using replicate assays at three concentration levels within a day ($n=9$, each concentration was prepared in triplicate). Inter-assay precision was assessed by using QC samples at the beginning, in between and at the end of the analytical sequence. Repeatability was performed by analyzing six replicates of QC samples. The precision and repeatability were calculated as the coefficient of variance (CV). The recovery was determined from each QC sample spiked with 10 μ L of mixed standard working solutions at high, medium, and low analytic concentrations respectively ($n=9$, each concentration was prepared in triplicate) before (A) and after (B) extraction. The concentration of post-extraction (A) and pre-extraction (B) was calculated by matrix-matched calibration curve, respectively. The recovery (%) = (A/B) $\times$ 100%.

Herein, the derivatization reaction yield of each SCFA was also calculated. Ninety microlitre of QC sample was spiked with 10 μ L of mixed standard working solutions at high, medium and low analyte concentrations, respectively ($n=9$, each concentration was prepared in triplicate) followed by derivatization. The concentrations of target compounds were back calculated by matrix-matched calibration curve after UHPLC-MS/MS quantitative analysis. These concentrations were actual concentrations. The concentrations according to principle of derivatization reaction were theoretical concentrations. The derivatization reaction yield was expressed as (actual concentration/theoretical concentration) $\times$ 100%.

Matrix effect was evaluated by QC-based standard samples (matrix-matched) at three concentration levels ($n=9$, each concentration was prepared in triplicate) and solvent-only standard samples at the same concentration. Their concentrations were back calculated by the corresponding calibration curves, respectively. The ratio between concentration of the QC-based standard samples and the solvent-only standard samples was used and final value was expressed as a percentage. Matrix effect (%) = (C_m/C_s) $\times$ 100%, where C_m and C_s respectively represent average concentration of the analyte of matrix and solvent.

2.8. Statistical analysis

To illustrate the change of the analytes in BALF levels, the data acquired were statistically analyzed by Independent-Sample *t* test in SPSS 19.0 soft-ware (Chicago, IL, USA). A value of $p < 0.05$ was considered to be statistically significant.

3. Result and discussion

3.1. OVA-induced asthma model

Compared with the normal mice, the OVA-induced mice were observed with marked infiltration of inflammatory cells in perivascular and peribronchial connective tissues (Fig. 1a). We also examined fibrosis and inflammation in the trachea tissues. Immunohistochemistry staining showed overexpression of α -SMA and P52 in the trachea tissues of the OVA-induced mice (Fig. 1b). Correspondingly, the serum concentration of IgE from OVA-challenged mice was remarkably higher than that of normal mice (Fig. 1c). In comparison with normal mice, the OVA-challenged mice showed significantly increases of the total cell counts and lymphocyte number ($p < 0.01$) in BALF (Fig. 1d, e). Taken together, these various observations suggested that OVA-induced asthma model was successfully established.

3.2. UHPLC-LTQ-Orbitrap based method development

After 3-NPH-based derivatization, the UHPLC-LTQ-Orbitrap method was optimized for the separation and detection of SCFAs in BALF. Firstly, chromatographic conditions were optimized through several trials to achieve desired separation, analytical time and symmetric peak shapes for derivatized SCFAs and IS in our preliminary experiment. The mobile phases consisting of water with 0.01% formic acid and acetonitrile have been demonstrated to be the optimum choice for target analytes [21]. The separation was carried out on a UHPLC column with 1.7 μ m particle size (Waters Acquity BEH C₁₈ column; 2.1 $\times$ 100 mm, 1.7 μ m) to reduce the analytical time. Different gradient elution programs were tried to achieve good separation efficiency at the flow rate of 0.35 mL/min. Under the optimized chromatographic conditions, most of the peaks could be separated within 13 min.

In theoretical, the derivatized analytes which contain nitrogen atom should be more easily ionized in positive mode. However, our study indicated that, compared with positive mode,

Table 1
Chromatographic and mass spectrometric characteristics of the analytes.

Compound	RT (min)	Parent ion (m/z)	Product ion(m/z)	Collision energy (eV)
Acetate	3.00	194.06	151.06	30
Propionate	4.89	208.07	165.07	30
Isobutyrate	6.44	222.10	179.09	30
Butyrate	6.68	222.10	179.09	30
2-Methylbutyrate	7.84	236.10	193.10	30
Isovalerate	8.08	236.10	193.10	30
Valerate	8.37	236.10	193.10	30
Caproate	9.84	250.10	207.12	30
IS: d4-Acetate	3.00	197.04	154.08	30

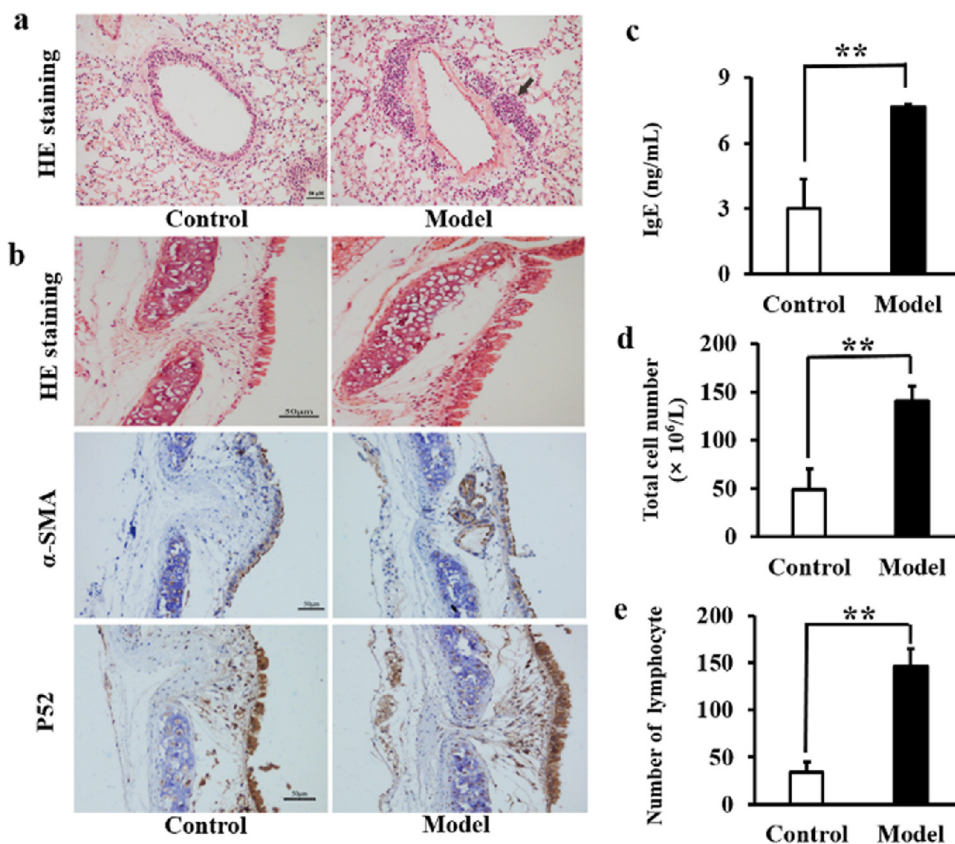


Fig. 1. OVA-induced murine airway inflammation. (a) Lung sections were stained with hematoxylin eosin (HE) and photographed using Olympus BX53 Microscope. One representative section per group was shown. (b) HE in the trachea and immunohistochemical staining of α -SMA, P52 in the trachea tissues were performed. The scale bars in a and b represent 50 μ m. (c) BALF serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA, Neobioscience Technology Company, Shanghai). (d) Total cell counts and (e) lymphocyte number were analyzed in BALF obtained from control and model group 24 h after the last saline or OVA exposure. Data are shown individually and as the mean $\pm$ SD, * p < 0.05, ** p < 0.01.

the use of negative mode made 3-NPH derivatized SCFAs much easier to be charged and prone to fragmentation (Figure S1). This result is in consistent with that of previous researches for the detection of phenylhydrazine derivatizations by LC–MS based methods [29–32]. Mechanically, the favorable distribution of negative charge in the conjugated system of electrons upon collision-induced fragmentation amplifies the signal for detection by mass spectrometers based on SRM [29]. Therefore, here the derivatized SCFAs are monitored in negative-ion mode. The parent ion and its specific product ion with high intensity for each transition were selected in SRM method. The derivatized SCFAs were monitored by product ion of $[M-H-43]^-$ from the different parent ions of respective analytes. And for the selected transitions, the corresponding CE (20, 25, 30, 35, 40 eV) were also tried to produce high sensitivity and good repeatability. As a result, all data were collected with a CE of 30 eV in SRM method for the detection of derivatized SCFAs (Fig. 2).

Procedural and instrumental blank contamination is a well-known problem in laboratories performing bioanalysis of different analytes. During the UHPLC-LTQ-Orbitrap method development, instrumental blank contamination was observed in solvent blank injections for acetate and caproate (Fig. 2). This contamination may arise from contaminated LC solvents or derivative reagents. Although several trials have been tried to elucidate the contamination source, the instrumental blank contamination was still observed with stable signals of acetate and caproate in solvent blank injections. Finally, their peak areas, 35,164 for acetate and 11,487 for caproate, were deducted before the calculation of standard curves and concentrations in samples.

3.3. Method validation

The quantitative metabolomics study requires a straightforward, comprehensive and reliable method. As displayed in Table 2,

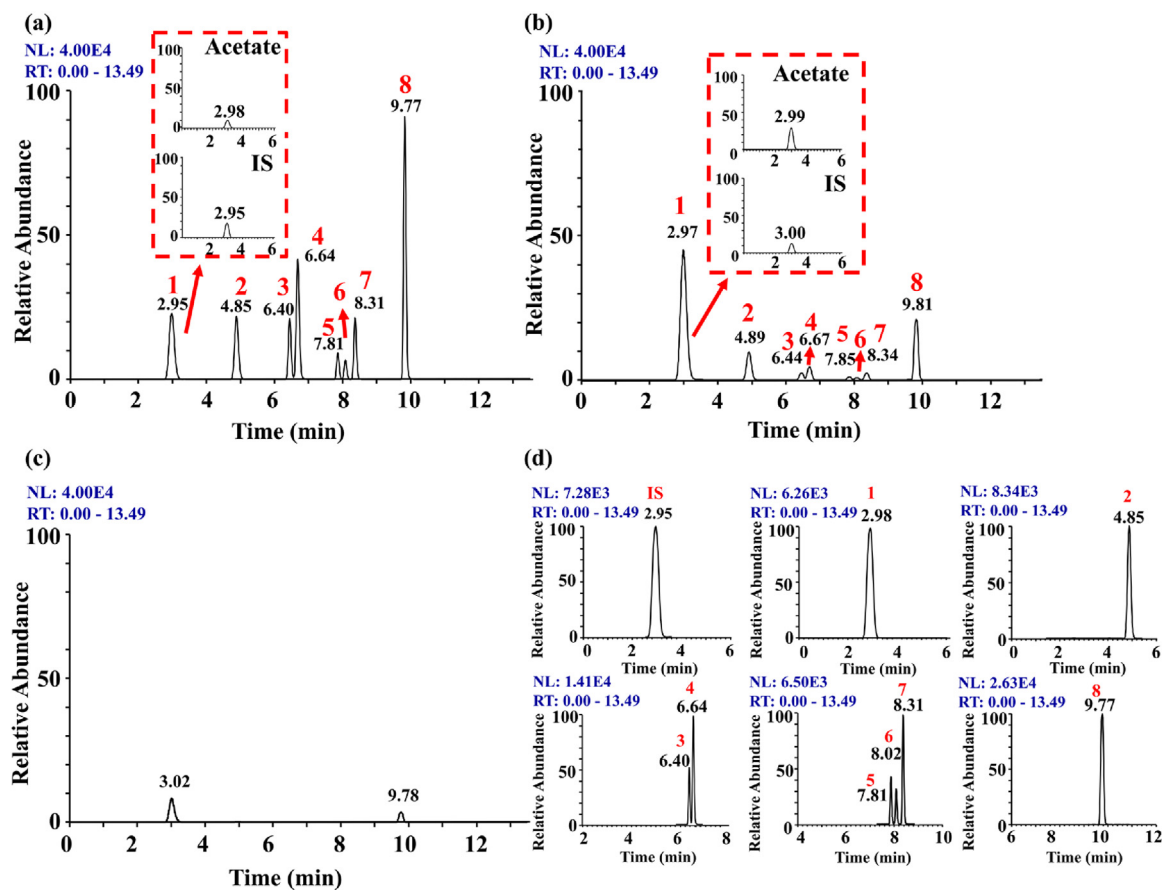


Fig. 2. UHPLC/SRM-MS of SCFAs standards (a), BALF sample (b), solvent blank (c) and extracted peaks of the targeted transitions (d). 1. Acetate, 2. Propionate, 3. Isobutyrate, 4. Butyrate, 5. 2-Methylbutyrate, 6. Isovalerate, 7. Valerate, 8. Caproate.

Table 2
Methodological observation: linearity.

Compound	Standard solution			BALF solution		
	Regression line	R^2	Linear range(nmol/L)	Regression line	R^2	Linear range(nmol/L)
Acetate	$y = 1.6577x - 0.1899$	0.9996	96–15360	$y = 1.6604x + 0.4184$	0.9994	24–19200
Propionate	$y = 3.6653x - 0.0982$	0.9998	9.6–7680	$y = 3.5375x + 0.0084$	0.9999	12–9600
Isobutyrate	$y = 3.5456x + 0.0477$	0.9996	96–3840	$y = 3.5396x + 0.0588$	0.9997	6–4800
Butyrate	$y = 3.8039x + 0.0384$	0.9997	0.096–7680	$y = 3.6954x + 0.1895$	0.9999	12–9600
2-Methylbutyrate	$y = 3.7120x - 0.0354$	0.9995	2.4–1920	$y = 3.5766x + 0.0167$	0.9999	3–2400
Isovalerate	$y = 2.8521x - 0.0436$	0.9995	2.4–1920	$y = 2.8080x + 0.0101$	0.9997	3–2400
Valerate	$y = 4.4031x - 0.0389$	0.9997	0.048–3840	$y = 4.2892x + 0.0260$	0.9999	6–4800
Caproate	$y = 4.1984x - 0.2487$	0.9997	96–15360	$y = 4.0609x + 0.4260$	0.9998	96–19200

“y”: SCFA/IS peak area ratio; “x”: nmol/L.

Table 3
Methodological observations: repeatability, intra- and inter-day precision, derivatization reaction yield, recovery and matrix effects.

Compound	Repeatability (CV, %)			Intra-day Precision (CV, %)			Inter-day Precision (CV, %)			Derivatization reaction yield (%)			Recovery (%)			Matrix effects (%)		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
Acetate	3.80	2.09	4.80	10.37	3.94	1.56	9.01	3.48	5.38	116.60	95.48	88.45	100.65	95.37	99.07	115.14	107.77	90.11
Propionate	3.17	1.97	4.79	3.42	3.15	1.03	2.64	2.86	4.82	97.22	99.65	89.65	95.30	94.23	95.61	107.16	103.23	92.06
Isobutyrate	1.62	2.64	5.62	3.02	2.96	0.49	2.53	2.93	4.89	97.94	111.93	87.73	95.31	94.91	98.72	104.39	100.19	88.53
Butyrate	3.69	1.70	6.51	2.32	0.94	1.22	4.10	2.03	6.60	88.65	100.84	90.50	94.48	95.18	96.31	105.45	103.66	89.99
2-Methylbutyrate	4.24	2.01	7.06	7.65	1.01	1.57	5.95	2.81	6.11	100.94	103.38	91.26	95.51	93.32	97.62	115.46	105.83	91.37
Isovalerate	1.40	3.51	6.08	6.62	1.90	1.33	3.73	2.99	5.51	94.43	98.44	90.46	92.18	94.01	98.58	102.16	101.51	90.96
Valerate	3.02	3.32	7.83	3.25	0.52	0.03	3.69	2.29	6.62	94.38	106.26	90.83	92.76	93.61	96.61	103.74	105.07	90.36
Caproate	2.69	0.91	5.53	1.92	0.88	1.14	3.49	2.64	6.60	81.24	101.76	91.91	94.63	93.04	97.49	114.30	107.46	93.26

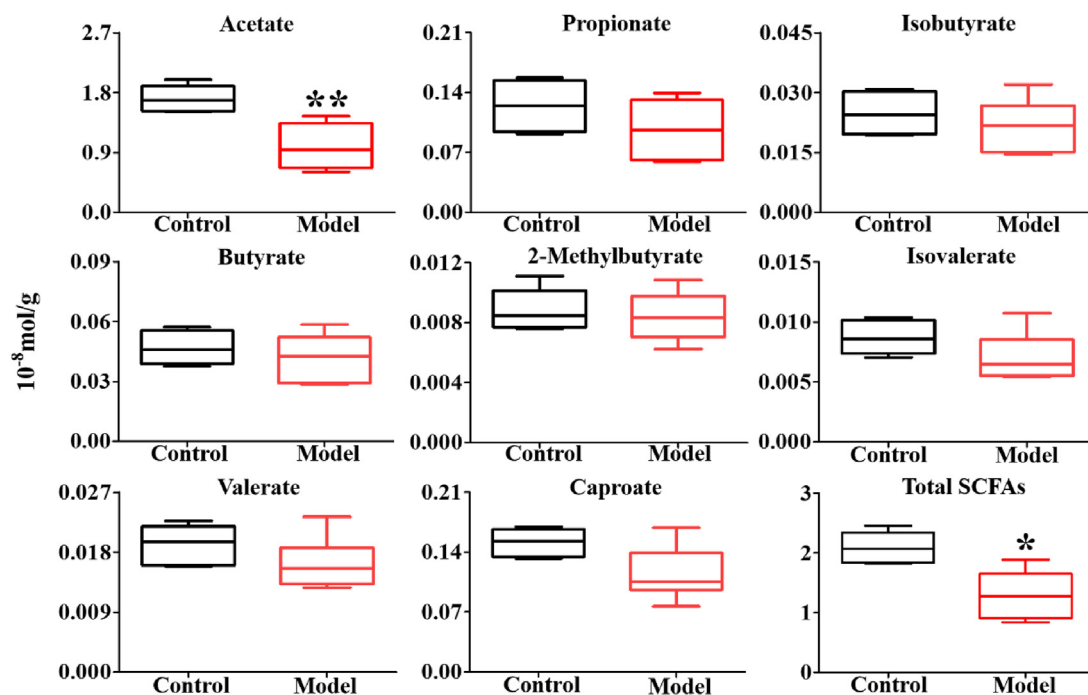


Fig. 3. SCFAs box plot of BALF. A two-tailed t test was applied to test for statistical significance. The values are the means $\pm$ SD; * $p < 0.05$, ** $p < 0.01$ for the control group vs the model group.

linearity were observed for all SCFAs in both of the solvent-only and matrix-matched calibration curves ($R^2 \geq 0.9992$). The lowest concentrations which would yield at least 10 times the signal-to-noise ratio, while giving CVs of the measured peak areas for individual analytes of $\leq 10\%$ ($n = 3$), were determined as the LLOQ. Herein, the LLOQ were determined to be varied from 3 to 24 nmol/L, which were used as the lowest concentrations for each target SCFAs' linear range. A comparison of the two sets of calibration curves for the target SCFAs was also performed (Figure S2). As shown, the two slopes of the two calibration curves for each SCFA were basically the same, indicating that the quantitative determination of SCFAs in the BALF samples was not affected by the sample matrix.

Method precision and repeatability were also checked by the QC samples (Table 3). As a result, the CV values of precision obtained from intra- and inter-day variability were 0.03–10.37% and 2.03–9.01%, respectively. Good repeatability was also demonstrated with CVs ranging from 0.91% to 7.83%.

Derivatization reaction yield of each SCFA with 3-NPH was also determined to assess the progress of derivatization reaction. As shown in Table 3, the derivatization reaction yields for target SCFAs were between 81.24%–116.60% ($n = 9$, each concentration was prepared in triplicate). The relative recoveries were determined to assess the accuracy of quantitation, which were found ranging from 90% to 110% for target SCFAs at three concentration levels ($n = 9$). Matrix effects for the developed method in quantitation of target SCFAs were ranging from 85% to 115% at three concentration levels ($n = 9$).

3.4. Method application

The LTQ-Orbitrap is a high-performance hybrid tandem instrument that can provide high mass resolution, high mass accuracy, and enhanced dynamic range with respect to mass accuracy [33,34]. We first reported the development of UHPLC-LTQ-Orbitrap-based quantitative analysis of 3-NPH-derived SCFAs in BALF. BALF is a valuable biosample in the study of respiratory diseases. Although the methodology of BALF collection has evolved to a

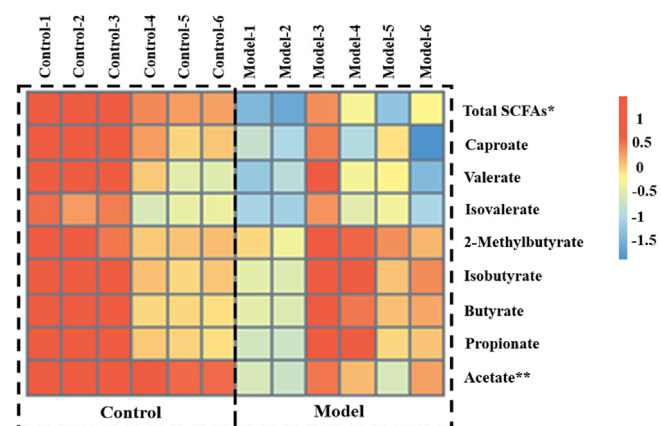


Fig. 4. OVA-induced allergic airway inflammatory SCFAs profile changes. BALF profiles of control and model groups were analyzed using SCFAs and expressed as a heatmap, denoting fold change over normalized means. Red squares indicate increases up to 1-fold, and blue squares indicate reductions up to 1.5-fold. * indicates statistical significance of model group as compared to control group, * $p < 0.05$, ** $p < 0.01$, $n = 6$ per group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

point where it is repeatable and responsive to inflammation, its use in molecular profiling studies is still limited. Herein, the developed method was successfully applied to quantify SCFAs in BALF samples from OVA-induced asthma mice and normal mice. As shown in Fig. 3, the total content of SCFAs in the BALF of the OVA-induced asthma mice were significantly decreased in comparison with that of normal mice, which were relevant to the increased prevalence of inflammatory disease [35]. Similarly, most of the individual SCFA also displayed a downward trend in asthma mice. Particularly, as a protective element against airway inflammation [3], acetate in asthma mice's BALF was significantly decreased. A heatmap was used for the comparative analysis of SCFAs profile in BALF between asthma and normal mice (Fig. 4). We found that recurrent OVA air-

way exposures led to concomitant decrease in acetate and total SCFAs as compared to controls.

Commensal bacteria always influence the development of asthma through the production and secretion of SCFAs [36,37]. As the best studied metabolites, SCFAs are mainly formed by the microbial fermentation of dietary fiber. Recent studies have also found that the human respiratory tract is also inhabited by niche-specific communities of bacteria. However, up to now, there is still no evidence to support that the bacteria of respiratory tract can produce SCFAs. In this study, in order to provide some valuable hints, we also applied the UHPLC-LTQ-Orbitrap method for quantitative profiling of SCFAs in feces, serum and lung tissue from asthma and normal mice (Figure S3-S5, Table S1-S3). As a result, we found that recurrent OVA airway exposures led to a similar decreasing trend for acetate and total SCFAs in these biosamples as compared to controls. In addition, we also compared the concentration levels of SCFAs in different biosamples (Table S4). The highest SCFAs level was found in feces, followed by the SCFAs level in lung tissue and serum. The concentration levels of SCFAs in BALF were much lower than that in serum. These evidences support that SCFAs were mainly produced in intestine and then were absorbed in blood. SCFAs in the lung should mainly come from the bloodstream. However, further study is still needed to demonstrate whether the bacteria of respiratory tract can produce SCFAs and what their substrates are.

4. Conclusion

In this paper, accurate quantification of SCFAs in BALF was performed based on UHPLC-LTQ-Orbitrap oriented 3-NPH-based derivatization, providing enhanced sensitivity and selectivity. Eight SCFAs with the wide concentration ranges of linearity were successfully quantified. The SCFAs-targeted method was then applied to determine the changed levels in BALF samples from OVA-induced asthma mice and normal controls. Our results indicated that most of the target SCFAs displayed a downward trend in asthma mice, of which acetate was significantly down regulated. In addition, the UHPLC-LTQ-Orbitrap oriented 3-NPH-based derivatization method was also applied to determine the concentration levels of SCFAs in feces, serum and lung tissue from asthma and normal controls. Similarly, acetate and the total SCFAs were significantly down regulated in asthma mice. The universality of our developed method in different samples gives us a chance to deduce the origin of SCFAs in lung, which is important for understanding the role of SCFAs in modulation of host metabolism and immunity.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jpba.2018.08.057>.

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