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ORIGINAL ARTICLE

# An integrative approach for compressive quality control of RespireAid™, a traditional Chinese medicine formula against SARS-CoV-2

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**Abstract**

**RespireAid™ (NRICM101) is an effective anti-SARS-CoV-2 traditional Chinese medicine formula and has been licensed as a drug or dietary supplement in Taiwan, Luxembourg, Australia, Singapore, Cambodia, Philippines, and Canada. In this study, we provided integrated quality control strategy to analyze the ingredient of RespireAid™. In addition, the lot-to-lot efficacy stabilities were also evaluated. We found that RespireAid™ comprised of monosaccharides and disaccharides (34.0%), maltodextrin (23.5%), inorganic elements and ash (12.2%), oligosaccharides and polysaccharides (11.4%), principal components (4.4%), moisture (4.0%), amino acids (3.5%), β-Cyclodextrin (0.25%), menthol (0.25%), and nucleotides (0.14%), while the remainder was unidentified (6.36%). This is the first time that the chemical composition of a complex traditional Chinese medicine was clarified using various analytical instruments. The lot-to-lot anti-oxidation and anti-inflammation efficacies of RespireAid™ were consistent, with average 50% scavenging concentrations of 0.22 ± 0.02 mg/mL and 5.76 ± 0.59 mg/mL, respectively. From a comprehensive quality control strategy point of view, RespireAid™, designed from a traditional Chinese medicine formula, displayed high quality, transparency, and efficacy. This integrated strategy provides a clear and reliable way to evaluate the quality of complex traditional Chinese medicines.**

*Keywords:* Compressive quality control, Integrative approach, Lot-to-lot consistency, Principal components, RespireAid™, Traditional Chinese medicine

## 1. Introduction

Coronavirus disease 2019 (Covid-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to a worldwide pandemic since December 2019. People infected with SARS-CoV-2 have the symptoms of dry cough, fever, fatigue, runny nose, nasal obstruction, diarrhea, and sore throat. Septic shock, acute respiratory distress syndrome, multiple organ failure, multisystem inflammatory syndrome in children, and death has been reported in severe cases [1,2]. According to data from the Covid-19 dashboard by the Center for System Science and Engineering of John Hopkins University, as of February 17, 2023, there have been 673, 650, 676 total Covid-19 cases with 6,860,275 deaths worldwide. In Taiwan, the total number of cases and deaths as of February 17, 2023 is 9,878,848 and 17,319, respectively. The Covid-19 pandemic has significantly influenced human health, economics, and social associations globally [3,4]. SARS-CoV-2, an RNA virus, has several variants ranging from Alpha (B.1.1.7) to Omicron (XBB.1.5) [5]. Antiviral drugs have suboptimal efficacy against SARS-CoV-2 because of the different variants. The new oral antiviral drugs Molnupiravir

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and Paxlovid are being used to treat the Omicron variant in Taiwan since 2022. However, there are many use restrictions, such as pregnancy, liver function, human immunodeficiency virus drug tolerance, bone development, and growth [6,7]. Therefore, East Asia countries have developed anti-SARS-CoV-2 drugs based on traditional Chinese medicine (TCM) theories. In TCM, the complexity of different herbal material components can provide multiple pathways for treatment; hence, TCM can possibly treat different SARS-CoV-2 variants.

In Taiwan, the first Covid-19 case was reported on January 21, 2020. The extensive TCM experience in Taiwan resulted in the National Research Institute of Chinese Medicine (NRICM) developing the formula called Taiwan Chingguan Yihau (NRICM101), which targeted virus infection, virus replication, and cytokine storms. The bench study revealed that NRICM101 blocked the viral spike protein to human angiotensin converting enzyme 2, inhibited the 3CL protease, and reduced the interleukin-6 and tumor necrosis factor-$\alpha$ cytokine storms. A bedside study showed that after intervention with NRICM101, the patient respiratory specimens tested negative three times for SARS-CoV-2 within nine days [8]. Taiwan has used TCM concentrated extracts since 1946. Different pharmaceutical companies were granted the license for NRICM101 in 2020 to expedite making the decoction formula for concentrated extracts.

RespireAid™ manufactured by Sun Ten Pharmaceuticals is the first concentrated extract licensed from NRICM101 since May 2020. Depending on the different regulatory policy by country, RespireAid™ has been licensed as a drug or dietary supplement in Taiwan, Luxembourg, Australia, Singapore, Cambodia, Philippines, and Canada. In Taiwan, RespireAid™ was licensed as legally approved for exportation on September 2, 2020, and received emergency use authorization as a drug on May 18, 2021. As shown in Table 1, RespireAid™ is composed of ten herbal materials including scutellariae radix, glycyrrhizae radix et rhizoma (processed), saposhnikoviae radix, herba, isatidis radix, menthae herba, trichosanthis fructus, mori folium, and magnoliae cortex. The formulation of RespireAid™ is instant soluble granules and the recommended dose is 10 g/day.

The quality control of products from natural sources faces severe challenges because of the complexity of components in herbal materials. Using HPLC to analyze the small organic components of herbal materials or TCM products is the most common strategy for quality control. Three dimensional HPLC quantum fingerprinting was used to analyze chlorogenic acid, *p*-coumaric acid, vitexin, and isovitexin in TCM Keteling capsules [9]. Nineteen marker components, including chlorogenic acid, baicalin, baicalein, wogonin, among others, were simultaneous analyzed for quality control of the herbal prescription Oncheong-Eum [10]. Besides advanced HPLC/DAD analysis, HPLC coupled with a linear ion trap-Orbitrap mass spectrometry (HPLC-LTQ-Orbitrap MS) was used to determine the 21 components of the Da-Huang-Xiao-Shi decoction [11]. Techniques other than chromatography, such as near-infrared spectroscopy (NIR), were applied for quality control of the oral herbal prescription Lanqin [12]. However, besides small organic components, other components in herbal materials also display biological activity. Polygonati rhizome polysaccharide has been reported to possess immune-regulation and anti-osteoporosis effects [13]. Oligosaccharides of *Morinda officinalis* are the quality markers of processed products [14]. Arginine-fructose complexes are well-known hypotensive compounds of *Panax ginseng* [15]. Our previous study applied LC/MS and ICP/MS to establish the fingerprint spectrum for the TCM preparation of Gan-Lu-Yin [16]. In addition to chemical analysis, we employed an efficacy stability test to evaluate the lot-to-lot quality of RespireAid™.

In this study, we applied integrated quality control strategy to analyze the ingredients of

*Table 1. Herbal materials in RespireAid™.*

| Herbal materials | Origin | Used part |
|---|---|---|
| Nepetae Herba | *Nepeta tenuifolia* Benth. | Dried aerial part |
| Scutellariae Radix | *Scutellaria baicalensis* Georgi | Dried root |
| Glycyrrhizae Radix et Rhizoma (processed) | *Glycyrrhiza uralensis* Fisch. | Dried root and rhizome |
| Saposhnikoviae Radix | *Saposhnikovia divaricate* (Turcz.) Schischk. | Dried root |
| Houttuyniae Herba | *Houttuynia cordata* Thunb. | Dried herb in flowering |
| Isatidis Radix | *Isatis indigotica* Fortune | Dried root |
| Menthae Herba | *Mentha haplocalyx* Briq. | Dried aerial part |
| Trichosanthis Fructus | *Trichosanthes kirilowii* Maxim. | Dried ripe fruit |
| Mori Folium | *Morus alba* L. | Dried leaf |
| Magnoliae Cortex | *Magnolia officinalis* Rehder et E. H. Wilson var. *biloba* Rehder et E. H. Wilson | Dried bark of trunk |

RespireAid™. This integrated strategy contains the various chemical ingredients and lot-to-lot efficacy stability test evaluations. Through this strategy, the comprehensive quality control of a TCM could be achieved.

## 2. Material and methods

### 2.1. Chemicals and reagents

All analytical standards were purchased from Sigma–Aldrich (Missouri, USA) and Fusol Material Co., Ltd. (Tainan, Taiwan). Reagents for HPLC and LC/MS/MS were purchased from Honeywell Research Chemicals (North Carolina, USA), Macron Fine Chemicals (Pennsylvania, USA), and Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside (SNP), and Griess reagent were purchased from Sigma–Aldrich (Missouri, USA). Purified deionized water was prepared using a Thermo Scientific Barnstead Easypure II (Massachusetts, USA).

### 2.2. Testing sample

RespireAid™ (lot number 20101933) was licensed from the NRICM101 formulation from the National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Executive Yuan, Taiwan, and manufactured by Sun Ten Pharmaceutical Co., Ltd., (New Taipei City, Taiwan) under Good Manufacturing Practice regulation. The herbal materials in RespireAid™ are listed in Table 1. At final stage of manufacturing process, 0.25% of menthol was added in RespireAid™. Maltodextrin and β-cyclodextrin were used as excipients.

#### 2.2.1. Sample preparation

The extraction solvents used depended on the analysis method. Approximately 1.0 g of sample was extracted using 20 mL of extraction solvent. For the 10 nucleotides, amino acid, DPPH and SNP-NO analyses, sample was extracted by the polar solvent, $H_2O$, through ultrasonication at 25 °C for 20 min [17]. For oligosaccharide and polysaccharide analyses, sample was extracted by $H_2O$ through ultrasonication at 25 °C for 20 min, repeated in 3 times. For principal component analysis, sample was extracted by 70% methanol through ultrasonication at 25 °C for 20 min. For monosaccharide and disaccharide analyses, sample was extracted by 50% ethanol through ultrasonication at 25 °C for 20 min. Moreover, for amino acid analysis, 2 mL of the sample solution was added 2 mL of 37% HCl and 10 mL of 6N HCl in a hydrolyzed bottle. The bottle was placed in an oven and hydrolyzed at 110 °C for 24 h. For inorganic elements analysis, 0.45 g of sample was put into a digestive vessel; then, 8 mL of nitric acid was added and the mixture was incubated for 10 min. Afterwards, the solution was digested using microwave digestive equipment (ETHOS EASY, Milestone, Sorisole, Italy). The digestion conditions were divided into two stages: stage 1 used 1,200 W max power, 100% power, 15 min ramp time, 175 °C, and hold for 5 min; stage 2 used 1,200 W max power, 100% power, 5 min ramp time, 200 °C, and hold for 15 min. After complete digestion and cooling to room temperature, the solution was filtered through a 0.45 μm filter membrane. Water was added to the filtrate to make the total volume 100 mL, and the resulting solution underwent ICP/MS analysis.

### 2.3. Small organic components analysis

#### 2.3.1. Principal components

The principal components analysis method was modified from a previous study [18]. The Waters HPLC system (Milford, Massachusetts, USA) comprised of a Waters 600 pump system, a Waters 2996 photodiode array detector, a Waters 717 plus autosampler, and a Sugai U-620 column oven (Wakayama City, Japan). A Cosmosil 5C18-MS-II reversed phase column (5 μm, 4.6 mm × 250 mm, Nacalai tesque, Japan) equipped with a Lichrospher RP-18 end-capped guard column (5 μm, 4.0 mm × 10 mm, Merck, Germany) was used as the stationary phase. The gradient elution of eluents A, B, and C (A: $H_2O$:$KH_2PO_4$:10% $H_3PO_4$, 1,000 mL:2.72 g:1 mL; B: $CH_3CN$; C: $H_2O$) was according to the following profile: 0–30 min, 90%–75% A and 10%–25% B; 30–40 min, 75%–65% A and 25%–35% B; 40–55 min, 65%–0% A, 35%–75% B, and 0%–25% C; 55–60 min, 75%–10% B and 25%–90% C; and 60–65 min, 0%–90% A, 10% B, and 90%–0% C. Gradient elution was used for the 3D fingerprint analysis and quantification of chlorogenic acid (9.9 min, 320 nm), epigoitrin (10.5 min, 240 nm), prim-*O*-glucosylcimifugin (21.8 min, 290 nm), liquiritin (26.8 min, 280 nm), rutin (27.5 min, 250 nm), rosmarinic acid (30.2 min, 320 nm), 5-*O*-methylvisammioside (30.8 min, 290 nm), quercitrin (32.5 min, 320 nm), hesperidin (33.7 min, 280 nm), baicalin (35.0 min, 280 nm), oroxylin A-7-*O*-glucuronide (40.6 min, 280 nm), wogonin-7-*O*-glucuronide (42.6 min, 280 nm), baicalein (50.7 min, 280 nm), glycyrrhizic acid (51.0 min, 250 nm), wogonin (55.2 min, 280 nm), pulegone (57.7 min, 250 nm), honokiol (59.6 min, 290 nm) and magnolol (61.5 min, 290 nm). The flow rate was

1 mL/min, and the column temperature was maintained at 35 °C.

#### 2.3.2. Nucleotides

Nucleotide analysis utilized the same experimental setup as described in section 2.3.1. The gradient elution of eluents A, B, and C (A: $H_2O$:$KH_2PO_4$:$K_2HPO_4$:10% $H_3PO_4$, 1,000 mL:1.36 g:1.74 g:1 mL; B: $CH_3OH$; C: $H_2O$) was according to the following profile: 0–25 min, 100%–60% A, 0%–20% B and 0%–20% C; 25–30 min, 60%–20% A, 20%–60% B and 20% C; and 30–45 min, 20%–100% A, 60%–0% B and 20%–0% C. The gradient elution was used for quantification of adenine, adenosine, cytidine, cytosine, guanosine, hypoxanthine, inosine, thymidine, uracil, and uridine. The UV absorbance of the nucleotides was measured at 260 nm with a flow rate of 1 mL/min. The column temperature was maintained at 35 °C.

### 2.4. Carbohydrate analysis

#### 2.4.1. Monosaccharide and disaccharide

The Waters HPLC system (Massachusetts, USA) comprised of a Waters 600 pump system, a Sedere Sedex 75 evaporative light scattering detector (Olivet, France), a Waters 717 plus autosampler, and a Sugai U-620 column oven (Wakayama City, Japan). An Asahipak NH2P-50 4E column (5 μm, 4.6 mm × 250 mm, Shodex, Japan) equipped with a Lichrospher RP-18 end-capped guard column (5 μm, 4.0 mm × 10 mm, Merck, Germany) was used as the stationary phase. The isocratic elution of $H_2O$:$CH_3CN$ (25:75) was used for quantification of fructose, galactose, glucose, sucrose, lactose, and maltose. The flow rate was 1 mL/min, and the column temperature was maintained at 35 °C.

#### 2.4.2. Oligosaccharide

The oligosaccharide analysis method was modified from a previous study [19]. The Waters HPLC system (Massachusetts, USA) comprised of a Waters 600 pump system, a Sedere Sedex 75 evaporative light scattering detector (Olivet, France), a Waters 717 plus Autosampler, and a Sugai U-620 column oven (Wakayama City, Japan). A Hypercarb column (5 μm, 4.6 mm × 100 mm, Thermo Fisher Scientific, USA) equipped with a Lichrospher RP-18 end-capped guard column (5 μm, 4.0 mm × 10 mm, Merck, Germany) was used as the stationary phase. The gradient elution of eluents A and B (A: 0.1% $CH_3COOH$; B: $CH_3OH$) was according to the following profile: 0–5 min, 100% A; 5–55 min, 100%–0% A and 0%–100% B; 55–60 min, 100% B; and 60–65 min, 0%–100% A and 100%–0% B. Stachyose was used as a standard to calculate the oligosaccharide content, following the previous study [19]. The flow rate was 1 mL/min, and the column temperature was maintained at 35 °C.

#### 2.4.3. Polysaccharide

The polysaccharide analysis method was modified from previous studies [20,21]. The Waters HPLC system (Massachusetts, USA) comprised of a Waters 600 pump system, a Waters 2414 refractive index detector, a Waters 717 plus autosampler, and a Sugai U-620 column oven (Wakayama City, Japan). A 5Diol-120-II column (7.5 mm × 300 mm, Waters, USA) was used as the stationary phase. The isocratic elution of $H_2O$:SDS:10% $H_3PO_4$ (1,000 mL:0.2 g:1 mL). Glucose was used as a standard to calculate the polysaccharide content, following the previous study [20,21]. The flow rate was 0.4 mL/min, and the column temperature was maintained at 35 °C.

### 2.5. Amino acid analysis

The amino acid analysis method was modified from the Chinese Pharmacopoeia 9th edition and a previous study [22]. The Shimadzu LC/MS/MS system (Kyoto, Japan) comprised of a CMB-20A system controller, a LC-20AD Pump, a SIL-20AC autosampler, an SPD-M20 PDA detector, a LCMS-8040 MS detector, and a CTO-30A column oven. A Biosil Aqu-ODS-W (5 μm, 4.6 mm × 250 mm, BIO-SIL, USA) coupled with a Synergi 4μ POLAR-RP 80A column (4 μm, 4.6 mm × 250 mm, Phenomenex, USA) was used as the stationary phase. The isocratic elution of eluents A and B (A: 5 mM heptafluorbutyric acid and 0.7% trifluoracetic acid; B: $CH_3CN$) was performed. For glycine, threonine, arginine, cysteine, histidine, serine, glutamine, lysine, alanine, glutamate, asparagine, proline, and aspartate analysis, the isocratic elution was 100% A. For isoleucine, tyrosine, tryptophan, valine, methionine, leucine, and phenylalanine analysis, the isocratic elution was 87% A and 13% B. The flow rate was 0.8 mL/min, and the column temperature was maintained at 40 °C.

### 2.6. Inorganic elements analysis

The inorganic elements analysis method was modified from a previous study [16]. An Agilent 7500a type ICP/MS system was used (Tokyo, Japan). The ICP/MS detection range was 2–260 amu, the RF forward power was 1,200 W, the sample depth was 7.8 mm, the carrier gas (Ar) flow rate was 1.12 L/min, and the extraction voltages were −120 V and −28 V.

### 2.7. Efficacy stability test

#### 2.7.1. DPPH scavenging assay

The DPPH-scavenging protocol was modified from a previous study [23]. Test samples were extracted with 70% methanol and serially diluted into different concentrations. DPPH ethanolic solution (200 μM) was mixed with the sample solutions in a 96-well microplate at room temperature for 20 min. The absorbance was evaluated by detecting the optical density of each well at 530 nm with a SPECTROstar Nano ELISA spectrophotometer (BMG Labtech, Germany).

#### 2.7.2. NO radical-scavenging assay

The NO radical-scavenging assay protocol was modified from a previous study [24]. SNP was used to evaluate the direct NO radical clearance of samples. Test samples were extracted with 70% methanol. The methanol was removed using a rotary evaporator and then the samples were re-dissolved in DMSO and serially diluted to different concentrations. The test solution was added to the sample volume of SNP solution (50 mM). The mixture was incubated at 37 °C for 5 h. Then, the supernatant was mixed with the Griess reagent. The absorption at 530 nm was measured with a SPECTROstar Nano ELISA spectrophotometer (BMG Labtech, Germany).

## 3. Results

### 3.1. Small organic components in RespireAid™

#### 3.1.1. Principal components

There are 10 herbal materials in RespireAid™. Except for finding no significant principal component in Trichosanthis Fructus, we analyzed at least 1 principal component from each herbal material. Fig. 1 displays the HPLC chromatograms of the principal components in RespireAid™. Baicalin from Scutellariae Radix was the most abundant principal component in RespireAid™, with a concentration of 26.85 ± 0.50 mg/g. The contents of the other components in mg/g were wogonin-7-*O*-glucuronide (6.40 ± 0.28), oroxylin A-7-*O*-glucuronide (3.37 ± 0.11), glycyrrhizic acid (2.79 ± 0.34), liquiritin (1.13 ± 0.19), hesperidin (0.54 ± 0.07), rosmarinic acid (0.51 ± 0.07), quercitrin (0.42 ± 0.10), chlorogenic acid (0.39 ± 0.04), baicalein (0.38 ± 0.04), 5-*O*-methylvisammioside (0.20 ± 0.02), prim-*O*-glucosylcimifugin (0.18 ± 0.02), honokiol (0.18 ± 0.05), wogonin (0.16 ± 0.03), magnolol (0.13 ± 0.02), epigoitrin (0.14 ± 0.01), rutin (0.09 ± 0.01), and pulegone (0.04 ± 0.01) (Table 2). 3D HPLC fingerprint of RespireAid™ was displayed in Fig. 2. The sum of the major principal components in RespireAid™ was approximately 43.87 mg/g, corresponding to 4.4% of the total mass.

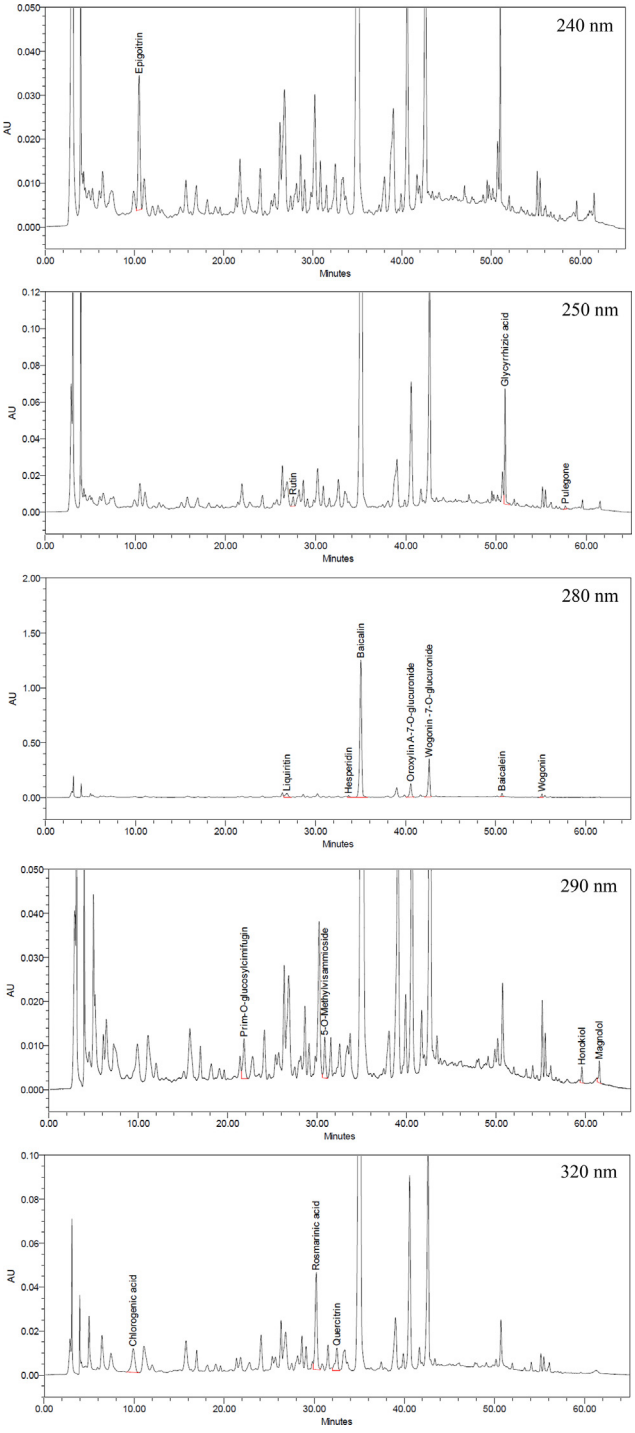


*Fig. 1. HPLC chromatogram of RespireAid™.*

*Table 2. Principal component content in RespireAid™.*

| Principal components | Herbal materials | Content (mg/g) |
|---|---|---|
| Hesperidin | Nepetae Herba | 0.54 ± 0.07 |
| Pulegone | | 0.04 ± 0.01 |
| Baicalin | Scutellariae Radix | 26.85 ± 0.50 |
| Baicalein | | 0.38 ± 0.04 |
| Wogonin | | 0.16 ± 0.03 |
| Oroxylin A-7-*O*-glucuronide | | 3.37 ± 0.11 |
| Wogonin-7-*O*-glucuronide | | 6.40 ± 0.28 |
| Glycyrrhizic acid | Glycyrrhizae Radix | 2.79 ± 0.34 |
| Liquiritin | et Rhizoma (Processed) | 1.13 ± 0.19 |
| 5-*O*-Methylvisammioside | Saposhnikoviae Radix | 0.20 ± 0.02 |
| Prim-*O*-glucosylcimifugin | | 0.18 ± 0.02 |
| Chlorogenic acid | Houttuyniae Herba | 0.39 ± 0.04 |
| Quercitrin | | 0.42 ± 0.10 |
| Epigoitrin | Isatidis Radix | 0.14 ± 0.01 |
| Rosmarinic acid | Menthae Herba | 0.51 ± 0.07 |
| Pulegone | | 0.04 ± 0.01 |
| Chlorogenic acid | Mori Folium | 0.39 ± 0.03 |
| Rutin | | 0.09 ± 0.01 |
| Honokiol | Magnoliae Cortex | 0.18 ± 0.05 |
| Magnolol | | 0.13 ± 0.02 |

#### 3.1.2. Nucleotides

Nucleotides in plants participate in many biochemical pathways, such as energy production and nucleic acid synthesis. As shown in Table 3, the five nucleotides with the highest contents in RespireAid™ are adenosine, uridine, guanosine, cytidine, and adenine. The sum of nucleotides in RespireAid™ was approximately 1.41 mg/g, corresponding to 0.14% of the total mass.

### 3.2. Carbohydrate in RespireAid™

#### 3.2.1. Monosaccharide and disaccharide

Monosaccharide, a single unit with a carbon chain of three to six carbons, is the simplest plant carbohydrate. Disaccharides are comprised of two monosaccharide units linked together by a glycosidic bond between a monosaccharide hydroxyl group and hydrogen. As shown in Table 4, sucrose, fructose, and glucose are the major monosaccharide and disaccharide components in RespireAid™, with a total amount of 340.43 mg/g, corresponding to 34.0% of the total mass. Mannitol, galactose, lactose, and maltose were not found in RespireAid™.

#### 3.2.2. Oligosaccharide and polysaccharide

Oligosaccharides are carbohydrates comprised of 3–10 monosaccharides, while polysaccharides are complex biomacromolecules formed by glycosidic linkage between monosaccharides. As shown in Table 4, the total contents of oligosaccharides and polysaccharides in RespireAid™ are 32.21 ± 0.94 mg/g and 81.91 ± 2.33 mg/g, corresponding to 3.2% and 8.2% of the total mass, respectively.

### 3.3. Amino acids in RespireAid™

Amino acids play a crucial role as the building blocks of protein. Each functional protein has a specific amino sequence. We analyzed the hydrolyzed form of amino acids in RespireAid™ using LC/MS/MS. As shown in Table 5, the five amino acids with the highest values in RespireAid™ are arginine, proline, glutamine, aspartate, and alanine. However, asparagine, glutamate, lysine, and tryptophan were not found. The sum of amino acid content in RespireAid™ was approximately 35.00 mg/g, corresponding to 3.5% of the total mass.

### 3.4. Inorganic elements in RespireAid™

Inorganic elements, defined as substances without both carbon and hydrogen, are important for the body's structure and function. The hazardous heavy metal content in RespireAid™, including lead (Pb, 0.164 ppm), cadmium (Cd, not detected), mercury (Hg, not detected), arsenic (As, 0.296 ppm), copper (Cu, 1.892 ppm), and chromium (Cr, 0.479 ppm), was analyzed using the ICP/MS quantitative method, and was found to conform with regulations in Taiwan, USA, and Europe (data not shown). Approximately seventy inorganic elements in RespireAid™ were analyzed using our ICP/MS semi-quantitative method, and the highest ten values were for potassium (K, 26,000 ppm), magnesium (Mg, 6,500 ppm), calcium (Ca, 1,800 ppm), sodium (Na, 1,600 ppm), aluminium (Al, 180 ppm), iron (Fe, 79 ppm), manganese (Mn, 63 ppm), strontium (Sr, 28 ppm), rubidium (Rb, 19 ppm), and beryllium (B, 17 ppm) (Fig. 3). The sum of inorganic elements in RespireAid™ was approximately 43,000 ppm, corresponding to 4.3% of the mass.

### 3.5. Efficacy stability of RespireAid™

To test the efficacy stability of RespireAid™, we collected seven different lots of RespireAid™ and tested the free radical scavenging effects with DPPH and SNP-NO scavenging assays. As shown in Fig. 4, the various lots of RespireAid™ displayed stable DPPH and SNP-NO scavenging effects with average $SC_{50}$ values of 0.22 ± 0.02 mg/mL and 5.76 ± 0.59 mg/mL, respectively.

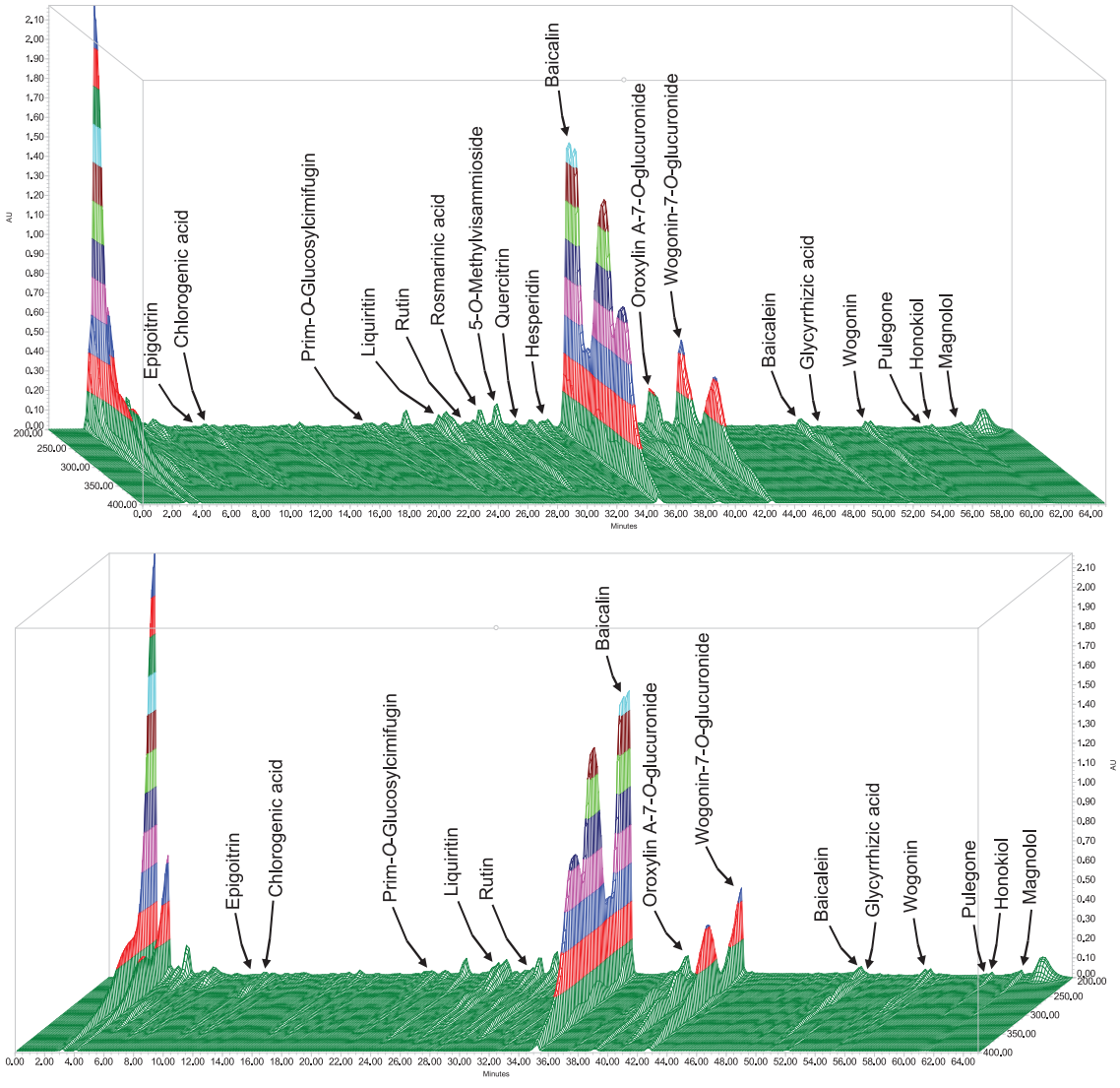


*Fig. 2. HPLC 3D fingerprint of RespireAid™.*

## 4. Discussion

Since 2020, research related to the treatment of Covid-19 using TCM has become popular because of the suboptimal therapeutic effects of western medicines. However, due to the complex components in herbal materials, most TCM quality control investigations focus on the active or principal components recorded in pharmacopoeia or databases.

*Table 3. Nucleotide content in RespireAid™.*

| Nucleotides | Content (mg/g) |
|---|---|
| Adenine | 0.15 ± 0.01 |
| Adenosine | 0.27 ± 0.02 |
| Cytidine | 0.17 ± 0.01 |
| Cytosine | 0.07 ± 0.01 |
| Guanosine | 0.21 ± 0.03 |
| Hypoxanthine | 0.08 ± 0.01 |
| Inosine | 0.05 ± 0.01 |
| Thymidine | 0.09 ± 0.02 |
| Uracil | 0.06 ± 0.01 |
| Uridine | 0.26 ± 0.03 |

*Table 4. Monosaccharide, disaccharide, oligosaccharide, and polysaccharide contents in RespireAid™.*

| Item | Content (mg/g) |
|---|---|
| Monosaccharide | |
| Fructose | 104.28 ± 1.21 |
| Galactose | Not Detected |
| Glucose | 82.61 ± 2.18 |
| Disaccharide | |
| Sucrose | 153.54 ± 3.12 |
| Lactose | Not Detected |
| Maltose | Not Detected |
| Oligosaccharide (Calculated as Stachyose) | 32.21 ± 0.94 |
| Polysaccharide (Calculated as Glucose) | 81.91 ± 2.33 |

*Table 5. Amino acid content (hydrolyzed form) of RespireAid™.*

| Amino acid | Ion pair for analysis | Content (mg/g) |
|---|---|---|
| Alanine | 90.00 > 44.15 | 1.67 ± 0.22 |
| Arginine | 175.00 > 70.10 | 9.36 ± 0.41 |
| Asparagine | 133.00 > 74.05 | Not Detected |
| Aspartate | 133.90 > 74.05 | 2.68 ± 0.10 |
| Cysteine | 122.00 > 59.00 | 0.20 ± 0.03 |
| Glutamine | 147.00 > 84.10 | 5.03 ± 0.77 |
| Glutamate | 148.00 > 84.10 | Not Detected |
| Glycine | 76.20 > 30.20 | 1.12 ± 0.31 |
| Histidine | 156.00 > 110.10 | 0.94 ± 0.05 |
| Isoleucine | 132.00 > 86.10 | 1.04 ± 0.07 |
| Leucine | 132.00 > 86.25 | 1.63 ± 0.08 |
| Lysine | 147.00 > 84.10 | Not Detected |
| Methionine | 149.90 > 56.10 | 0.17 ± 0.02 |
| Phenylalanine | 166.00 > 120.10 | 1.25 ± 0.09 |
| Proline | 116.00 > 70.10 | 6.16 ± 0.43 |
| Serine | 106.00 > 60.10 | 0.74 ± 0.09 |
| Threonine | 120.00 > 74.10 | 1.00 ± 0.02 |
| Tryptophan | 205.00 > 188.00 | Not Detected |
| Tyrosine | 182.00 > 91.10 | 0.49 ± 0.01 |
| Valine | 118.00 > 72.15 | 1.52 ± 0.33 |

The chemical compositions of anti-Covid-19 TCM include Shenfu decoction (4 herbal materials), Xuanbai Chengqi decoction (4 herbal materials), and Shufeng Jiedu capsule (8 herbal materials). These compositions, obtained from the Traditional Chinese Medicine System Pharmacology Database and Analysis Platform (TCMSP), are classified as herbal principal components, such as ginsenodies from *P. ginseng*, baicalin from *Scutellaria baicalensis*, and sennosides from *Rheum officinale* [1,25,26]. Moreover, various LC/MS/MS techniques are frequently used as analysis tools to determine the complex composition of TCM. Lianhua Qingwen capsule, a popular TCM for the treatment of Covid-19, was analyzed for 104 and 185 compounds with HPLC-Q Exactive/Orbitrap/MS coupled with GC/MS and UPLC/HRMS, respectively. A total of 217 chemical constituents of the anti-Covid-19 TCM Huashi Baidu prescription were analyzed using UPLC-Q-TOF/MS. However, the total amounts of all these principal components were approximately 1–5% of the entire TCM formulation [27–29]. As we described earlier, the total amount of the 19 principal components from the 10 herbal materials in RespireAid™ was only 4.4% of the composition (Table 2). Due to the limitation of analysis standards and methods, certain peaks in the HPLC chromatogram were unidentified and need to be determined using advanced instrumentation in future studies.

Secondary herbal metabolites, including terpenoids, flavonoids, polyphenols, alkaloids, saponins, and coumarins, are well-known as active ingredients in TCM, such as baicalin, the sesquiternoid in *S. baicalensis* or ephedrine, and the alkaloid in *Ephedra sinica.* However, other components, such as nucleotides or primary herbal metabolites (e.g., carbohydrates), also have crucial roles in TCM. Nucleotides are monomeric units of deoxyribonucleic acid and ribonucleic acid. Nucleotides are medically applied as antiviral agents, for example against hepatitis and human immunodeficiency virus [30]. Carbohydrates, including monosaccharides, disaccharides, oligosaccharides, and polysaccharides, are the basic energy source for the human body, and also have physiological functions. In addition to the well-known benefit for human gut health, oligosaccharides were reported to have antiviral properties. Wang et al., demonstrated that sulfated chitooligosaccharides could significantly decrease influenza A virus titers [31]. Oligosaccharides from *Porphyridium* sp were also reported to have anti-SARS-CoV-2 main protease activity [32]. Polysaccharides from herbal materials are well-known for their immunomodulation effects.

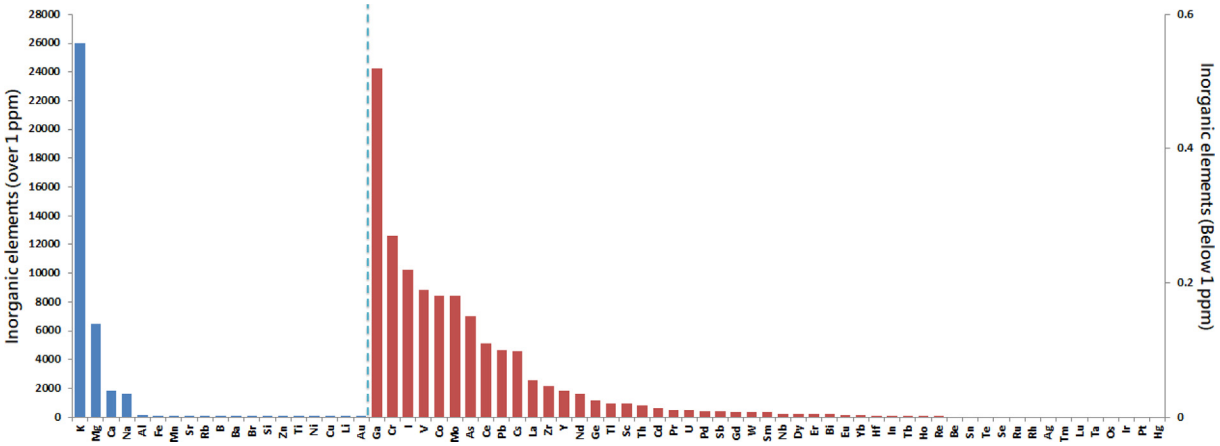


*Fig. 3. Inorganic element content of RespireAid™.*

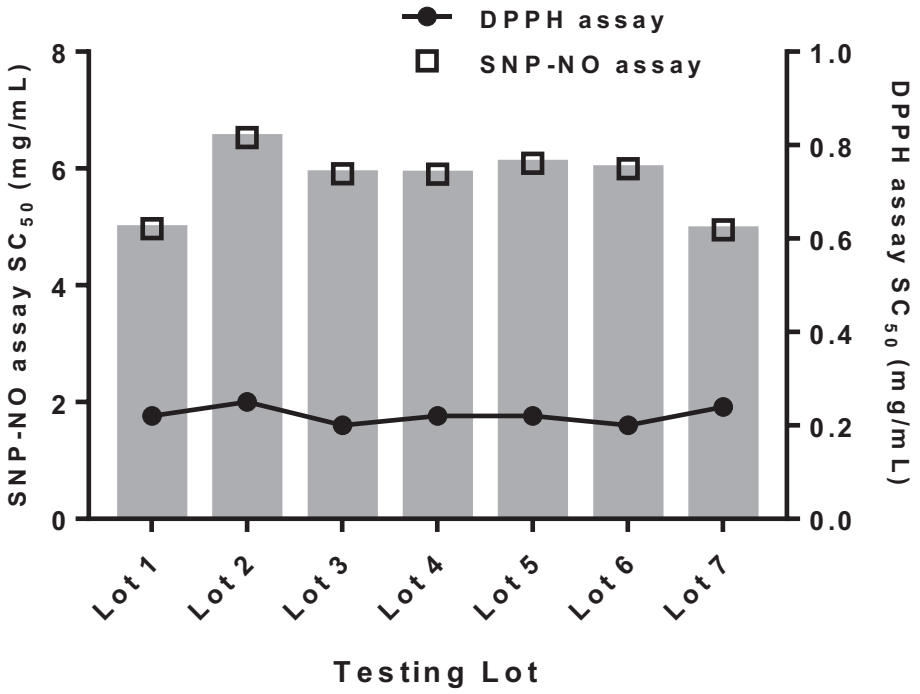


*Fig. 4. Efficacy stability of RespireAid™. The scavenging concentration of 50% is denoted $SC_{50}$. The lot numbers of 7 lots of RespireAid™ were 20100536, 20101933, 20111731, 20120734, 20121434, 21072637, and 21100436.*

Polysaccharides from seaweed were found to inhibit SARS-CoV-2 propagation by blocking viral entry [33]. Together, these data suggest that different types of carbohydrates could have an important role in the prevention of SARS-CoV-2, beyond the action of just the organic principal components. In RespireAid™, maltodextrin and β-cyclodextrin are used as excipients. Our analysis methods for monosaccharide, disaccharide, oligosaccharide, and polysaccharide could not analyze the maltodextrin and β-cyclodextrin excipient content due to the experimental design. Hence, the data in Table 4 is only in reference to the herbal extracts. Menthol, a hydrophobic and volatile monoterpene compound of Menthae Herba, has also been reported for the anti-SARS-CoV-2 activities. Evidence displayed that menthol showed remarkable ACE2 (angiotensin converting enzyme 2) inhibition effects, which could decrease the binding of SARS-CoV-2 spike protein and host cell ACE2 receptor [34]. The molecular docking study displayed the same results. The nsp10-nsp16 complex of coronavirus act the critical role in virus replication. Molecular docking results displayed that menthol had significant effects against SARS-CoV-2 nsp10-nsp16 protein complex, resulting in the prevention of SARS-CoV-2 infection [35,36]. Based on the therapeutic theory of traditional Chinese medicine, the multiple components displayed the multiple therapeutic targets. In RespireAid™, each principal component displayed specific activities against SARS-CoV-2. Baicalin, a key flavonoid of Scutellariae Radix, inhibited main protease, papain-like protease and RNA-dependent RNA polymerase of SARS-CoV-2. Quercitrin, a glycoside formed from the flavonoid quercetin of Houttuyniae Herba, interacted with SARS-CoV-2 spike protein, blocking virus infection. Glycyrrhizic acid, a triterpene glycoside of Glycyrrhizae Radix et Rhizoma, inhibited the binding of SARS-CoV-2 spike protein and host cell ACE2 receptor [37]. Hesperidin, a flavanone glycoside of Nepetae Herba, inhibited host cell ACE2 receptor internalization and SARS-CoV-2 virus replication and prevented excess inflammation [38]. Chlorogenic acid, a polyphenolic compound of Houttuyniae Herba and Mori Folium, inhibited SARS-CoV-2 $3CL^{pro}$ main protease [39]. Honokiol, a lignan of Magnoliae Cortex, was reported for blocking SARS-CoV-2 entry ability [40].

Balanced dietary supplements, such as amino acids, can improve immunity against viral infection and alleviate infection symptoms. As shown in Table 5, the three amino acids with highest values in RespireAid™ were found to be arginine, proline, and glutamate. In addition to the general functions of amino acids in the human body, Al-Kuraishy, et al., demonstrated that arginine could inhibit SARS-CoV-2 infections through different

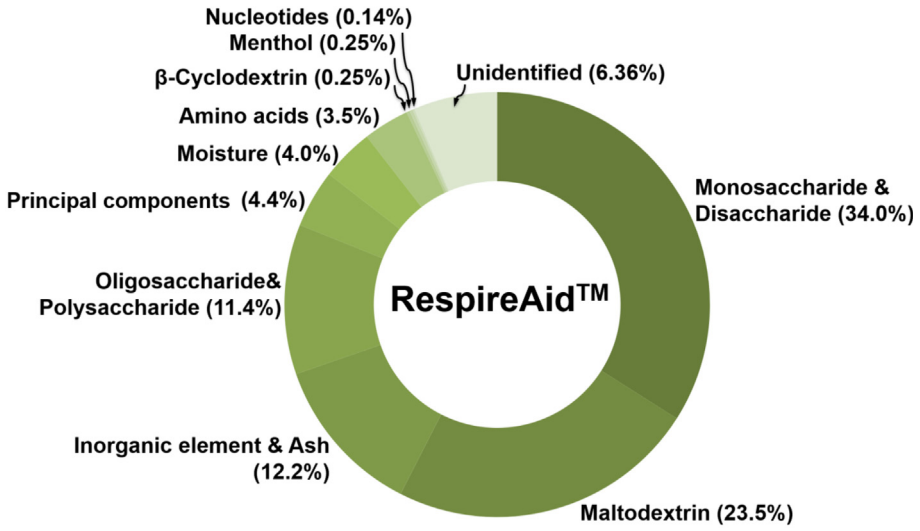


*Fig. 5. Determined ingredients of RespireAid™. The percentages of maltodextrin, ash, moisture, β-Cyclodextrin, and menthol were obtained from the manufacturer.*

mechanisms. In Covid-19 patients, glutamine supplementation could alleviate SARS-CoV-2-induced cytokine storms, including those of interleukin-1β and tumor necrosis factor-α [41,42]. Besides the common secondary metabolites in TCM, amino acids, such as arginine, also play an anti-viral role in RespireAid™ and should be monitored. In this study, we also screened the 70 inorganic elements in RespireAid™ using an ICP/MS semi-quantitative method. The most abundant inorganic elements in RespireAid™ were potassium (26,000 ppm), magnesium (6,500 ppm), and calcium (1,800 ppm). Compared to the calcium content in dietary food supplements, such as the common banana fruit, the content in RespireAid™ was found to be much lower [43]. Therefore, administration of RespireAid™ would not influence the dietary potassium-to-magnesium ratio.

The chemical analysis of active components is the common tool to determine the lot-to-lot stabilities of drugs. In routine manufacturing quality control of RespireAid™, baicalin and glycyrrhizic acid were used as targets to perform stability tests. The average amounts of baicalin and glycyrrhizic acid in the seven lots shown in Fig. 4 were 28.69 ± 0.30 mg/g and 2.74 ± 0.09 mg/g, respectively (data not shown). In addition to active components analysis, we applied an efficacy stability test for RespireAid™ because of its complex chemical composition. Oxidative stress and nitric oxide free radicals are two important mechanisms in SARS-CoV-2 infections; therefore, we used *in-vitro* anti-oxidation and anti-inflammation experiments to monitor the efficacy stabilities of RespireAid™ [44,45]. The efficacy stability results were the same as those of the active components; RespireAid™ displayed fine lot-to-lot stabilities of its active components and efficacy. Moreover, our preliminary data demonstrated that RespireAid™ would not influence the cytochrome P450 (CYP) enzyme gene expression, including *CYP2A6, CYP2B6, CYP3A4,* and *CYP3A5* (data not shown). We will discuss potential herb–drug interactions in a future study.

This work performs the quality control of TCM for the first time using integrated strategy. The ingredient content of RespireAid™ is shown in Fig. 5. Totally, approximately 93.64% of the ingredients in RespireAid™ were analyzed. Due to analysis method limitations and selections, the remaining 6.36% of unidentified ingredients will be analyzed in the future. Probable unidentified ingredients include principal components, vitamins, and oligosaccharides and polysaccharides. This integrated strategy could be applied to confirm the quality of TCM in the future. Through the comprehensive quality control strategy, from the whole-fingerprint point of view, RespireAid™, designed from TCM, displayed high quality, transparency, and efficacy.

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### Conflict of interest

The authors have declared no conflict of interest.

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