ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

Studies on the Composition and Glycoside Hydrolysis of Glycosides of 3-Oxo-ionol in Loquat Leaves

Yuan Lin[1], Yuchen Zhou[1], Yongjiang Wang[2], Li Zhang[1,*], Shengxiong Huang[1,2,*]

[1] Sichuan University of Science & Engineering, Zigong, Sichuan 643000, China

[2] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

[*] Corresponding Author: sxhuang@mail.kib.ac.cn; zhangli@suse.edu.cn

Abstract

3-Oxo-ionol, a flavor compound with low natural abundance, serves as a distinctive flavoring agent in various products such as wine and cigarettes. To find an alternative resource of 3-oxo-ionol, six 3-oxo-*α*-ionol glycosides and their analogs were isolated from loquat leaves. These compounds were identifies as (6*R*,7*E*,9*R*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (1), (*Z*)-6-[9-(*β*-*D*-glucopyranosyloxy)butylidene]-5,1,1-trimethyl-4-cyclohexen-3-one (2), (6*R*,7*E*,9*S*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (3), blumeol C glucoside (4), (6*S*,9*R*) roseoside (6), and (6*S*,9*S*) roseoside (7). Among them, compounds 2 and 4 were isolated from loquat leaves for the first time. Comparison of acid and enzymatic hydrolysis revealed that acid hydrolysis is more practical for hydrolyzing 3-oxo-*α*-ionol glycosides. After optimizing acid concentrations, compound 3 reached a hydrolysis efficiency of 35.6% with 2.0 M HCl. This method was applicable to other 3-oxo-*α*-ionol glycosides, offering a solution for the production of 3-oxo-ionol. This study not only presented a method for obtaining 3-oxo-ionol, but also expanded its production strategies, facilizing further research and application of these valuable compounds.

Keywords

3-oxo-ionol; 3-oxo-ionol Glycosides; Loquat Leaves; Acidic Hydrolysis; Enzymatic Hydrolysis.

1. Introduction

3-Oxo-ionol, a natural fragrance substance, acts as a distinctive flavoring agent in wine and cigarettes, etc [1,2,3]. As a fragrance enhancer, 3-oxo-ionol can offer a uniform release, thereby improving the quality of wine and tobacco products. During the combustion of cigarettes, 3-oxo-*α*-ionol could undergo thermal dehydration, releasing the flavor component megastigmatrienone [2,3]. Additionally, 3-oxo-ionol can serve as a precursor to synthesize flavoring 3-oxo-ionol ester compounds [4]. However, the natural abundance of 3-oxo-ionol is low, limiting its commercial applications as a fragrance enhancer. Given the significant economic value of 3-oxo-ionol, Daisuke and colleagues investigated the synthesis of 3-oxo-*α*-ionol, which required 12 steps and resulted in a total yield of 8.5% [5].

3-oxo-*α*-ionol glycosides are abundant in various plant species, including Loquat (*Eriobotrya Japonica*) [6], Oak (*Quercus spinosa*) [7] and *Eucommia ulmoides* [8], etc. Among them, loquat products have been used historically as both medicine and food for thousands of years [9], and its leaves are particularly rich in China, Japan, and India. The abundant 3-oxo-*α*-ionol glycosides found in loquat leaves may serve as alternative raw materials for the acquisition of fragrance substance 3-

ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

oxo-*α*-ionol. Therefore, we aimed to explore an efficient hydrolysis method for 3-oxo-*α*-ionol glycosides to yield 3-oxo-ionol.

In this study, six 3-oxo-*α*-ionol glycosides and their analogs (**1** – **4**, **6** and **7**) were isolated from loquat leaves, with compounds **2** and **4** being reported as isolated from loquat leaves for the first time. To determine an efficient method for hydrolyzing 3-oxo-*α*-ionol glycosides, compound **3** was selected for both acid hydrolysis and enzymatic hydrolysis experiments. A comparison of enzymatic and acid hydrolysis methods revealed that acid hydrolysis was more practical. By optimizing various acid concentration, the hydrolysis efficiency of **3** reached 35.67% with 2.0 M HCl. This method was universally applicable to other 3-oxo-*α*-ionol glycosides, providing a solution to produce 3-oxo-ionol.

2. Materials and Methods

2.1 Materials

Leaves were collected from loquat planted in Kunming Institute of Botany, Chinese Academy of Sciences. *α*-Amylase was purchased from Sichuan Weikeqi Biological Technology Co., Ltd. Hesperidinase was purchased from Shanghai Ruiyong Biotechnology Co., Ltd.

2.2 Extraction and Isolation

Dried loquat leaves (25 kg) were ground into powder. The loquat leaf powder was sequentially extracted using petroleum ether, dichloromethane, ethyl acetate, n-butanol, and methanol. The 1 kg of n-butanol extract separated using a silica gel chromatography column, employing gradient elution with a mobile phase of dichloromethane: methanol in the following ratios: 50:1, 20:1, 10:1, 5:1, 1:1, and 0:1. Fractions, containing similar components verified by TLC, were combined, resulting in a total 6 fractions. Fr.C (159.0 g) was subJected to gradient elution using a silica gel chromatography column, with a mobile phase of dichloromethane: methanol in the ratios of 1:0, 20:1, 10:1, and 0:1, resulting 4 fractions (Fr.C1 to Fr.C4). Fr.C3 (72.0 g) was subJected to gradient elution using an MCI chromatography column, with a mobile phase of methanol: water in the ratios of 1:5, 1:4, 3:10, 7:20, 2:5, 1:2, and 1:0, resulting 7 fractions (Fr.C3-1 to Fr.C3-7). Fr.C3-5 (1.6 g) and Fr.C3-6 (2.9 g) were isolated on a semi-preparative high-performance liquid chromatography (HPLC) system Hitachi Chromaster 5430 with a column (YMC-Triart C_{18}, 5 μm, 250 × 10 mm I.D.). The elution program involved a gradient of methanol and water as follows: 40% methanol from 0 to 10 min; 50% methanol from 10 to 20 min; 55% methanol from 20 to 25 min; 100% methanol from 25 to 30 min; and 40% methanol from 30 to 35 min. Detection was performed at a wavelength of 240 nm, resulting in the isolation of compounds **1** (t_R = 18.2 min, 350 mg), **2** (t_R = 19.4 min, 65mg), **3** (t_R = 20.1 min, 510 mg), **4** (t_R = 21.0 min, 430 mg) and **5** (t_R = 7.6 min, 5mg). After the identification of compound **5** as a mixture by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, compound **5** was subJected to a elution program (with a mobile phase of methanol and water) as follows: 25% methanol from 0 to 10 min; 30% methanol from 10 to 20 min; 35% methanol from 20 to 25 min; and 25% methanol from 25 to 30 min. Detection was performed at a wavelength of 240 nm, yielding compound **6** (t_R = 19.7 min, 2 mg) and compound **7** (t_R = 20.8 min, 1 mg). ^{1}H and ^{13}C NMR spectra of compounds **1** – **7** were acquired using a Bruker Avance III-600 MHz NMR spectrometer and compared with literature data to confirm their structures.

To verify the product of acid hydrolysis reaction, we separated it using a preparative liquid chromatography system. The mobile phase consisted of methanol and water, with the following elution program: 60% methanol from 0 to 10 min; 65% methanol from 10 to 20 min; 70% methanol from 20 to 25 min; and 100% methanol from 25 to 30 min. Detection was performed at a wavelength of 240 nm, resulting in the isolation of product **a** (t_R = 15.0 min) and product **b** (t_R = 15.3 min).

2.3 Enzymatic Hydrolysis of Compound 3

The experiments referred to the enzymatic hydrolysis method by Matsumoto [10]. Compound **3** (0.1 mg) was dissolved in 2 mL of phosphate-citrate buffer (pH 4.0) with 10 mg of *α*-amylase or ammonium acetate buffer (pH 4.0) with 10 mg of hesperidinase, respectively. Each mixture was

ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

stirred in a water bath at 40°C (α-amylase) or 50°C (hesperidinase) for 16 hours. The reactions were quenched by adding an equal volume of acetonitrile, then the mixtures were centrifuged at 12,000 rpm for 10 minutes to collect the supernatant.

To optimize efficiency of enzymatic hydrolysis, experiments were performed with buffers at pH 3.0, 4.0, and 5.0. All supernatants were analyzed using HPLC (Agilent 1290) with a YMC-Triart C_{18} column (5 μm, 250 × 4.6 mm I.D.). The mobile phase consisted of acetonitrile and water, with a gradient of 10%-100% acetonitrile from 0 to 20 minutes, 100% acetonitrile from 20 to 24 minutes, and 10% acetonitrile from 24 to 28 minutes. The products were detected at a wavelength of 240 nm.

2.4 Acidic Hydrolysis Experiments

The acid experiments were performed according to the Masayuki's acidic hydrolysis method [11]. Compound **3** (1.0 mg) was dissolved in 1 mL of methanol, then 1 mL of 2.0 M HCl was added. The mixture was refluxed at 80°C in an oil bath for 3 hours. Then the solution was neutralized to pH 7.0 using 1 mL of 2.0 M NaOH solution and extracted with ethyl acetate. The ethyl acetate layer was evaporated and dissolved in 1 mL methanol. The analysis method was consistent with that in the enzymatic hydrolysis experiments. To optimize efficiency of acid hydrolysis, the HCl concentration in the solution varied at 0.1 M, 0.5 M, 1.0 M, 1.5 M, 2.0 M, and 2.5 M (the reaction volume at 2 mL). Additionally, the same procedures were performed with compounds **1**, **2**, and **4** at a condition of 2.0 M HCl to evaluate the universality of acid hydrolysis condition.

3. Results and Analysis

3.1 Structure Identification

The loquat leaves were successively extracted using petroleum ether, dichloromethane, ethyl acetate, n-butanol, and methanol. Six compounds (Figure 1) were isolated from the n-butanol portion and identified by mass spectrometry (MS), NMR data and by comparison with literature as (6*R*,7*E*,9*R*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (**1**), (*Z*)-6-[9-(*β*-*D*-glucopyranosyloxy)butylidene]-5,1,1-trimethyl-4-cyclohexen-3-one (**2**), (6*R*,7*E*,9*S*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (**3**), blumeol C glucoside (**4**), (6*S*,9*R*) roseoside (**6**), (6*S*,9*S*) roseoside (**7**). Compounds **2** and **4** were isolated from loquat leaves for the first time.

(6*R*,7*E*,9*R*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (1): Brown oil; MS (m/z): $[M + Na]^+$ 393.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 202.1, 165.9, 138.2, 128.8, 126.1, 102.5, 78.1, 78.0, 77.0, 75.3, 71.5, 62.7, 56.8, 48.3, 37.1, 28.0, 27.6, 23.8, 21.0; ^{1}H NMR(600 MHz, CD_3OD) δ_H 5.88 (1H, s, H-4), 5.78 (1H, dd, J = 15.5, 6.5 Hz, H-8), 5.64 (1H, dd, J = 15.5, 9.3 Hz, H-7), 4.40 (1H, m, H-9), 4.35 (1H, d, J = 7.8 Hz, H-1′), 3.82 (1H, dd, J = 11.9, 2.4 Hz, H_A-6′), 3.65 (1H, dd, J = 11.9, 5.5 Hz, H_B-6′), 2.68 (1H, d, J = 9.3 Hz, H-6), 2.43 (1H, d, J = 16.7 Hz, H_A-2), 2.05 (1H, dd, J = 16.7 Hz, H_B-2), 1.94 (3H, s, H-11), 1.29 (3H, d, J = 6.4 Hz, H-10), 1.03 (3H, s, H-12), 1.01 (3H, s, H-13). [12]

(*Z*)-6-[9-(*β*-*D*-glucopyranosyloxy)butylidene]-5,1,1-trimethyl-4-cyclohexen-3-one (2): Brown oil; MS (m/z): $[M + Na]^+$ 393.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 201.9, 159.6, 144.8, 129.6, 129.2, 103.9, 78.2, 77.9, 77.2, 75.2, 71.7, 62.8, 53.6, 41.9, 37.9, 28.3, 28.2, 25.1, 22.0. ^{1}H NMR(600 MHz, CD_3OD) δ_H 5.94 (1H, t, J = 6.7 Hz, H-7), 5.90 (1H, s, H-4), 4.38 (1H, d, J = 7.8 Hz, H-1′), 3.99 (1H, q, J = 6.2 Hz, H-9), 3.86 (1H, dd, J = 12.1, 1.9 Hz, H_A-6′), 3.67 (1H, dd, J = 12.0, 5.3 Hz, H_B-6′), 2.64 (1H, m, H_A-8), 2.56 (1H, m, H_B-8), 2.31 (1H, d, J = 17.1 Hz, H_A-2), 2.29 (1H, d, J = 4.7 Hz, H_B-2), 2.27 (3H, s, H-11), 1.28 (3H, d, J = 6.2 Hz, H-10), 1.19 (3H, s, H-12), 1.19 (3H, s, H-13). [13]

ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

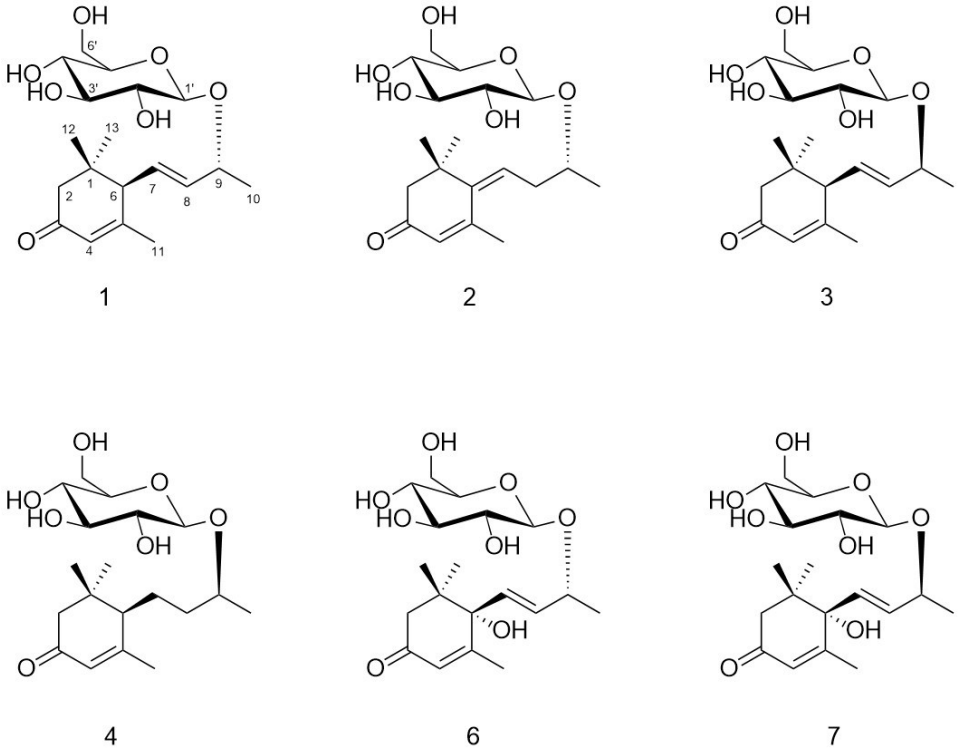

Figure 1. Structure of compounds **1** - **4**, **6** and **7** identified in loquat leaves

(6*R*,7*E*,9*S*)-9-hydroxy-4,7-megastigmadien-3-one 9-O-*β*-*D*-glucopyranoside (3): Brown oil; MS (m/z): $[M + Na]^+$ 393.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 202.0, 165.7, 137.0, 131.2, 126.2, 101.2, 78.3, 78.2, 74.9, 74.8, 71.7, 62.8, 56.9, 48.5, 37.2, 28.0, 27.4, 23.9, 22.2. ^1H NMR(600 MHz, CD_3OD) δ_H 5.89 (1H, s, H-4), 5.75 (1H, dd, J = 15.3, 9.4 Hz, H-8), 5.59 (1H, dd, J = 15.3, 7.5 Hz, H-7), 4.48 (1H, m, H-9), 4.29 (1H, d, J = 7.8 Hz, H-1′), 3.85 (1H, dd, J = 12.3, 2.2 Hz, H_A-6′), 3.63 (1H, dd, J = 11.9, 6.2 Hz, H_B-6′), 2.70 (1H, d, J = 9.4 Hz, H-6), 2.48 (1H, d, J = 16.7 Hz, H_A-2), 2.06 (1H, d, J = 16.7 Hz, H_B-2), 1.98 (3H, s, H-11), 1.29 (3H, d, J = 6.5 Hz, H-10), 1.03 (3H, s, H-12), 0.99 (3H, s, H-13). [6]

Blumeol C glucoside (4): Brown oil; MS (m/z): $[M + Na]^+$ 395.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 202.4, 169.9, 125.4, 104.0, 78.2, 77.8, 77.6, 75.3, 71.7, 62.8, 52.7, 48.1, 37.4, 37.4, 29.0, 27.5, 26.7, 25.0, 22.0. ^1H NMR(600 MHz, CD_3OD) δ_H 5.81 (1H, s, H-4), 4.32 (1H, d, J = 7.9 Hz, H-1′), 3.85 (1H, dd, J = 11.6, 1.9 Hz, H_A-6′), 3.82 (1H, m, H-9), 3.66 (1H, dd, J = 11.9, 5.2 Hz, H_B-6′), 2.49 (1H, d, J = 17.5 Hz, H_A-2), 2.05 (3H, s, H-11), 1.99 (1H, d, J = 6.8 Hz, H_B-2), 1.97 (1H, d, J = 5.7 Hz, H-6), 1.81 (1H, m, H_A-7), 1.69 (1H, m, H-8), 1.68 (1H, m, H_B-7), 1.25 (3H, d, J = 6.3 Hz, H-10), 1.10 (3H, s, H-12), 1.02 (3H, s, H-13). [14]

(6*S*,9*R*) Roseoside (6): Brown oil; MS (m/z): $[M + Na]^+$ 409.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 201.2, 167.3, 135.3, 131.5, 127.2, 102.7, 80.0, 78.1, 78.0, 77.3, 75.2, 71.6, 62.8, 50.7, 42.4, 24.7, 23.4, 21.2, 19.6. ^1H NMR(600 MHz, CD_3OD) δ_H 5.98 (1H, d, J = 15.6 Hz, H-7), 5.87 (1H, s, H-4), 5.73 (1H, dd, J = 15.6, 7.3 Hz, H-8), 4.54 (1H, dq, J = 6.7, 6.5 Hz, H-9), 4.27 (1H, d, J = 7.8 Hz, H-1′), 3.85 (1H, dd, J = 11.7, 1.7 Hz, H_A-6′), 3.63 (1H, dd, J = 11.9, 6.1 Hz, H_B-6′), 2.61 (1H, d, J = 17.1 Hz, H_A-2), 2.17 (1H, d, J = 14.7 Hz, H_B-2), 1.94 (3H, s, H-11), 1.29 (3H, d, J = 6.4 Hz, H-10), 1.04 (3H, s, H-12), 1.02 (3H, s, H-13). [15]

(6*S*,9*S*) Roseoside (7): Brown oil; MS (m/z): $[M + Na]^+$ 409.2; ^{13}C NMR (150 MHz, CD_3OD) δ_C 201.3, 167.1, 133.8, 133.7, 127.1, 101.2, 80.0, 78.4, 78.2, 74.9, 74.6, 71.7, 62.8, 50.7, 42.4, 24.7, 23.5, 22.2, 19.6. ^1H NMR(600 MHz, CD_3OD) δ_H 5.98 (1H, d, J = 15.6 Hz, H-7), 5.87 (1H, s, H-4), 5.73 (1H, dd, J = 15.6, 7.3 Hz, H-8), 4.42 (1H, dq, J = 6.3, 5.7 Hz, H-9), 4.34 (1H, d, J = 7.8 Hz, H-1′), 3.85 (1H, dd, J = 11.7, 1.7 Hz, H_A-6′), 3.63 (1H, dd, J = 11.9, 6.1 Hz, H_B-6′), 2.61 (1H, d, J = 17.1

ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

Hz, H_A-2), 2.17 (1H, d, J = 14.7 Hz, H_B-2), 1.94 (3H, s, H-11), 1.29 (3H, d, J = 6.4 Hz, H-10), 1.04 (3H, s, H-12), 1.02 (3H, s, H-13). [16]

3.2 The Hydrolysis of 3-oxo-*α*-ionol Glycosides

3.2.1 Hydrolysis Conditions of Compound 3

Compound **3**, the most abundant compound isolated from loquat extract, was selected for glycosidic hydrolysis experiments. To investigate the methodologies for glycosidic hydrolysis, both enzymatic and acidic hydrolysis were chosen for evaluation. In the enzymatic hydrolysis experiment, *α*-amylase and hesperidinase were tested with compound **3** under three pH conditions: 3.0, 4.0 and 5.0. LC-MS analysis of the enzymatic hydrolysis experiments indicated that compound **3** was hydrolyzed by *α*-amylase at pH 4.0, resulting in a yield of 5% (Figure 2). The low hydrolysis efficiency of compound **3** renders it unsuitable for practical application. In the acidic hydrolysis experiment, hydrochloric acid at a final concentration of 1.0 M was initially evaluated. The acid hydrolysis experiments of compound **3** showed two distinct products, labeled as **a** and **b** (Figure 2). Product **b** corresponded to the product obtained from enzymatic hydrolysis. Under the condition of 1M HCl, the hydrolysis efficiency was low, yielding 6.46% for product **a** and 5.81% for product **b.**

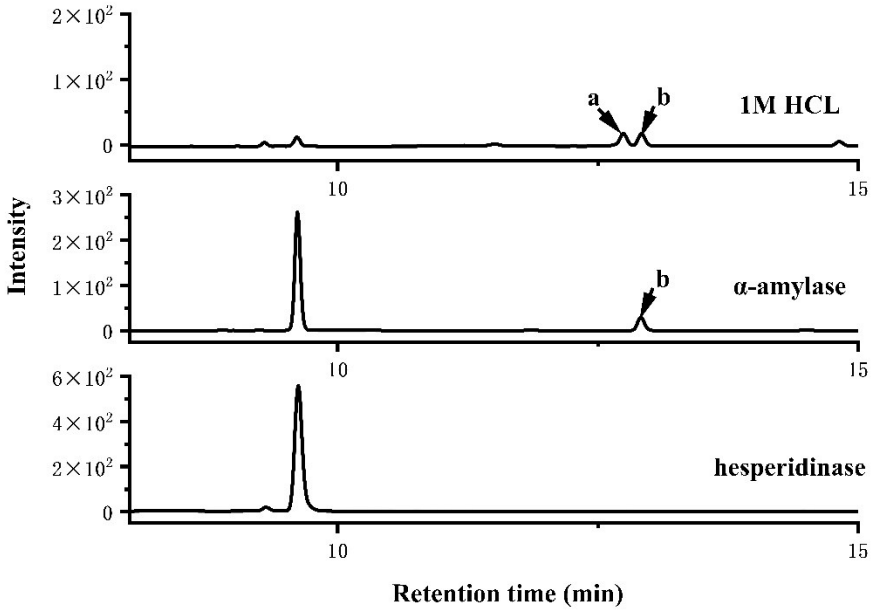

Figure 2. Enzymatic and acid hydrolysis of compound **3**

Since product **b** corresponded to the product observed in the enzymatic hydrolysis with *α*-amylase, we speculated that product **a** is 3-oxo-*β*-ionol and product **b** is 3-oxo-*α*-ionol. This phenomenon might be caused by the high-temperature, strong-acid conditions, which induced the isomerization of 3-oxo-*α*-ionol and 3-oxo-*α*-ionol glycosides.

To enhance hydrolysis efficiency, we conducted six experiments with varying concentrations of hydrochloric acid. As a result, compound **3** was hydrolyzed at hydrochloric acid concentrations ranging from 0.5M to 2.5M. Similar to the hydrolysis of compound **3** with 1M HCl, these hydrolysis yielded two products. The yields were as follows: 4.18% of **a** and 3.55% of **b** for 0.5M HCl; 6.56% of **a** and 5.97% of **b** for 1M HCl; 16.95% of **a** and 15.43% of **b** for 1.5M HCl; 19.33% of **a** and 16.34% of **b** for 2M HCl; 9.41% of **a** and 8.00% of **b** for 2.5M HCl (Figure 3). These results indicated that refluxing with 2M HCl at 80°C for 3 hours was the optimal condition to hydrolyze compound **3**.

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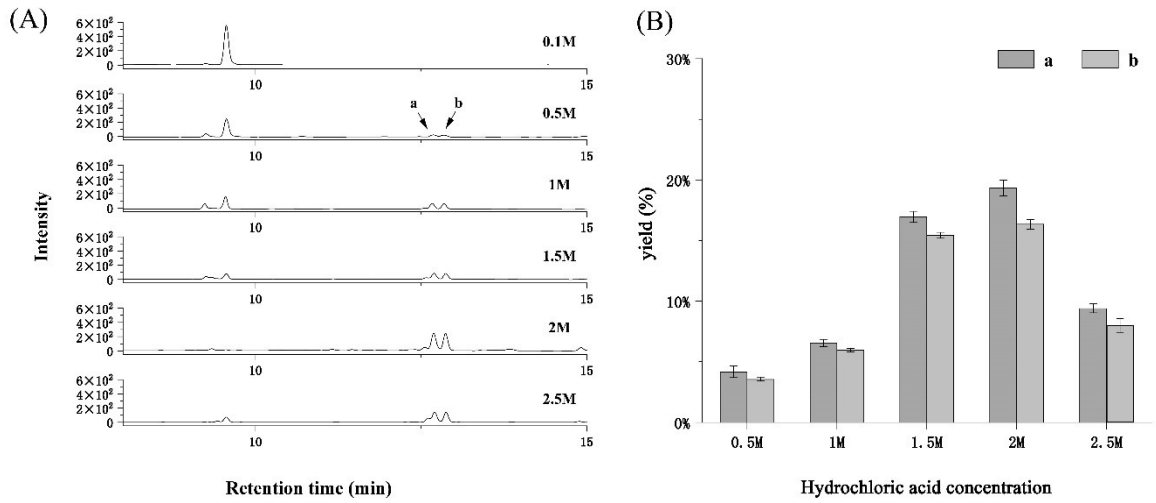

Figure 3. Gradient acid hydrolysis of compound **3**. (A) HPLC data for the hydrolysis of compound **3** under different hydrochloric acid concentrations. (B) Yields of hydrolysis products of compound **3** under different hydrochloric acid concentrations.

3.2.2 Acid Hydrolysis of Three Other Compounds

To assess the efficiency of the optimal acid hydrolysis condition for other glycosides of 3-oxo-*α*-ionol, we conducted hydrolysis experiments on compounds **1**, **2**, and **4** (1.0 mg each) under identical conditions. LC-MS analysis confirmed that all reactions successfully produced the corresponding aglycones, with hydrolysis efficiencies of 19.81%, 29.21%, and 53.32%, respectively (Figure 4). These results indicated that the optimized method was generally effective for hydrolyzing other glycosides of 3-oxo-*α*-ionol.

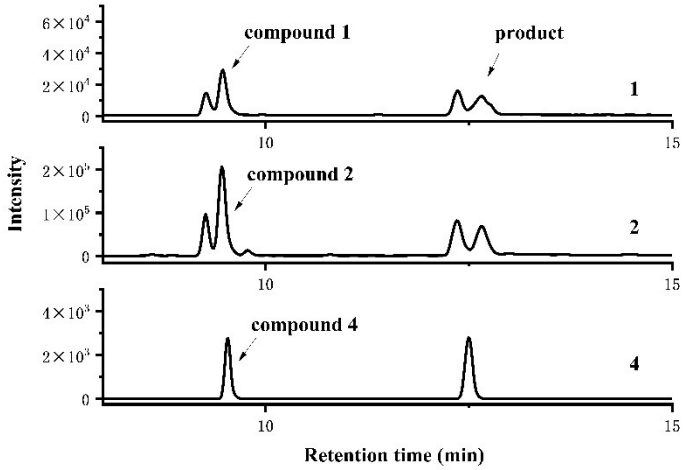

Figure 4. Acid hydrolysis of compounds **1**, **2** and **4**

4. Conclusion

3-Oxo-ionol and its analogs exhibit significant commercial value as flavoring agents in various products such as wine and cigarettes. In this study, six 3-oxo-*α*-ionol glycosides and their analogs (**1** - **4**, **6** and **7**) were isolated from the loquat leaves. Among them, compounds **2** and **4** were isolated from loquat leaves for the first time. To investigate methods for obtaining the aglycones of 3-oxo-*α*-ionol glycosides, compound **3** were hydrolyzed by both enzymatic and acid hydrolysis experiments. The low enzymatic hydrolysis efficiency made it not suitable for the efficient acquisition of aglycone 3-oxo-*α*-ionol. In contrast, acid hydrolysis using 2M HCl at 80°C for 3 hours achieved high hydrolysis

ISSN: 2414-1895 DOI: 10.6919/ICJE.202504_11(4).0012

efficiency, successfully converting the 3-oxo-*α*-ionol glycosides into their aglycone forms. This method is proved to be a practical approach for obtaining aglycones from other 3-oxo-*α*-ionol glycosides and their analogs, thereby diversifying and enhancing the production strategies of flavoring agents. These advancements will facilitate further research and applications of these valuable compounds. References.

Acknowledgments

This work was supported by Yunnan Revitalization Talent Support Program “Yunling Scholar” Project.

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