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Identification of Clenbuterol by MALDI-TOF Mass Spectrometry

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Matrix-assisted laser desorption ionization time-of-flight mass spectrometry（MALDI-TOF-MS）was used to analyze macromolecular compounds. The present study focused on how to use MALDI for determination of small molecules such as clenbuterol, and how to explore the matrix to improve the sensitivity. The macromolecular 1,2, 3,4-tetrakis(3',4'-hydroxyphenyl) thiophene matrix not only absorbed the laser energy but also caused sample desorption ionization. At the same time, it did not produce interfering ions of low molecular weight. Moreover, when perfluoro C_{60} was added to the matrix, sensitivity was improved.

Keywords: matrix-assisted laser ionization; clenbuterol; matrix; sensitivity.

INTRODUCTION

The MALDI-TOF systems have evolved considerably since the first commercial version was introduced by Shimadzu in 1988 [1-3]. Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) [4-6] and large organic molecules (such as polymers, dendrimers and other macromolecules) [7,8], which tend to become fragile and fragmented when ionized by more conventional ionization methods. The strength of MALDI-TOF-MS lies in the ability to generate a molecule profile representing a large number of molecules expressed by the specific organism tested. This introduces a high level of molecule multiplex testing with results in minutes. The molecule profile can be used for microbial identification, detecting specific strains, and detection of antibiotic-resistant markers [9, 10].

The MALDI process starts by adding the sample to a metal target slide. A matrix solution is then applied to the sample, resulting in formation of crystals. A focused laser beam, either in the UV or IR range, can “evaporate” compounds from the solid phase; the sample is ionized with a laser pulse where the absorption of high energy by the matrix crystals results in vaporization of the molecules that are accelerated by a voltage gradient through the time-of-flight tube. The velocity of the molecules through the time-of-flight tube is inversely proportional to the size and charge of the molecule. As the molecules separate based on size and charge, smaller molecules reach the detector first, followed by larger molecules, resulting in the creation of a series of peaks named spectra. It was said that a MALDI-TOF instrument in a shared proteomics facility can easily be set up to handle hundreds of analyses per day [11-13].

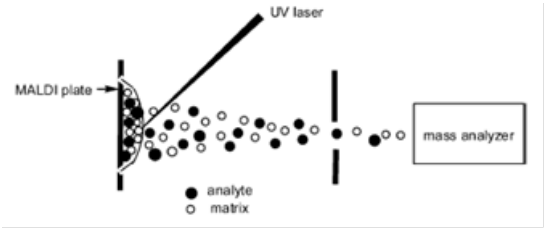

Fig. 1. Scheme graph of MALDI-TOF MS

The matrix is the core of the MALDI study. The matrix should meet the following conditions: (1) to be miscible with the measured object, diluting the analyte, reducing the force between the molecules, and preventing the formation of molecular clusters; (2) to be stabilized under the vacuum existing; (3) to strongly absorb the laser light source; (4) to be able to protect the sample towards the laser irradiation, the energy transfer to the analyte not directly destroying the structure of the analyte; (5) to provide protons by proton transfer to promote analyte ionization; (6) to provide the volume flow, where analyte molecules are thrown. Different types of compounds should be generally used in different matrices. For MALDI, the selection of matrix has a significant impact on the final results of the experiments; the choice of

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the matrix is also related with the formation of the fragment ions. By now, there is still no perfect theoretical guidance on how to choose the matrix for the different samples [14,15].

Clenbuterol is a class of animal drugs which are β-agonists; there are several kinds of drugs such as ractopamine, clenbuterol, etc. When clenbuterol is added to the food, carcass lean meat of animals can be increased with reduced food use and the meat be sold with reduced costs. However, clenbuterol will produce side effects to human body; an excessive intake will produce abnormal physiological responses such as nausea, dizziness, muscle tremors, palpitations, increased blood pressure and other symptoms of poisoning. Ministry of Agriculture has issued documents (No.176, No.193 Notice and No.1519 Ordinance) prohibiting the use of β-agonists as feed additives. Clenbuterol in animal feed is often detected by GC-MS, HPLC, ELISA and LC-MS/MS [16,17].

When the molecular weight of the sample is less than 400, the matrix (a small-molecule compound) commonly used in MALDI-TOF MS will produce ionization, leading to a mass spectrum of the small-molecule compounds existing in a large number of hetero-peaks that cannot be described. Hetero-peaks have a very strong mass discrimination effect, lead to small-molecule compounds which are not easily ionized to molecular ions. So the main purpose of the study is to find a matrix which is not only able to absorb laser energy but also can ionize the sample, at the same time not generating interfering ions of low molecular weight. In this way we will be able to take advantage of MALDI-TOF MS analysis of different types of small-molecule compounds [18].

EXPERIMENTAL

Materials

Matrix: 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene (provided by the Institute of Chemistry, Chinese Academy of Sciences); α-cyano-4-hydroxy cinnamic acid (CCA) (Aldrich Co.); perfluoro C_{60} (provided by the Institute of Chemistry, Chinese Academy of Sciences).

Solvents: ethanol, tetrahydrofuran (THF) (Beijing Chemical Factory); Milli Q ultrapure water.

Ionization reagents: sodium chloride; trifluoroacetic acid (Beijing Chemical Factory).

Samples: ractopamine; salbutamol; clenbuterol; terbutaline (Beijing Antai advanced technology development Co.).

Laboratory instruments and parameter settings

Instrument: Bruker autoflex MALDI-TOF MS equipped with a semiconductor laser, wavelength 355 nm, maximum power 200 μj, attenuation 25% -45% of the laser operation. The delay leads and reflected working mode were used, delay time was 35000 ns, delayed extraction voltage 14.5 Kv, acceleration voltage 19 Kv, reflector voltage 20 kV. 150 superimposed scan signals formed the mass spectrum.

Configuration of sample, matrix, and ionized solution

Ractopamine, salbutamol, clenbuterol and terbutaline samples were dissolved in aqueous ethanol solution (ethanol:water=1:1), the concentration of the solution was 10^{-2} mol/L; 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene or α-cyano-4-hydroxy cinnamic acid (CCA) matrix was weighed, dissolved in aqueous ethanol solution (ethanol:water=1:1), the concentration of the solution was 10^{-3} mol/L; sodium chloride or trifluoroacetic acid ionizing solutions were weighed, dissolved in aqueous ethanol solution (ethanol:water=1:1), the concentration of the solution was 10^{-5} mol/L.

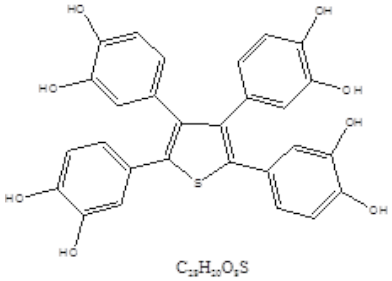
Sample preparation

The sample solution, 3 μL of the matrix solution, and 1 μL of ionizing reagent (if necessary) were thoroughly mixed, a 0.5 μL droplet was placed on a clean stainless steel sample target, and after the solvent was evaporated, the crystalline sample was sent to the mass spectrometer for analysis.

RESULTS AND DISCUSSION

Matrix choice

As shown in Fig. 2, 1,2,3,4-tetrakis (3', 4'-hydroxyphenyl)-thiophene contains eight phenolic hydroxyl groups which can provide a large number of protons in the ionization process; the protons are transferred to the sample molecules, so that sample ionization occurs.



$C_{28}H_{20}O_8S$
Exact Mass: 516.09
Mol. Wt.: 516.52
m/e: 516.09 (100.0%), 517.09 (32.6%), 518.09 (6.5%), 518.08 (4.4%), 519.09 (1.5%)
C, 65.11; H, 3.90; O, 24.78; S, 6.21

Fig. 2. 1, 2, 3, 4 – tetrakis (3', 4'-hydroxyphenyl) thiophene structure

Furthermore, 1, 2, 3, 4 – tetrakis (3',4'-hydroxy-phenyl) thiophene has a relatively large molecular weight (516.09) which can be effectively reduced to produce a large number of hetero-peaks in the region of Da less than 400.

The UV absorption diagram of 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene is shown in Fig. 3. UV laser absorption by the sample molecules is at 355 nm.

When terbutaline was determined by the MALDI-TOF mass spectrum, the concentration of the matrix solution was 10^{-3} mol/L, terbutaline sample solution concentration was 10^{-2} mol/L, sodium chloride concentration was 10^{-5} mol/L, solvent: ethanol:water =1:1.

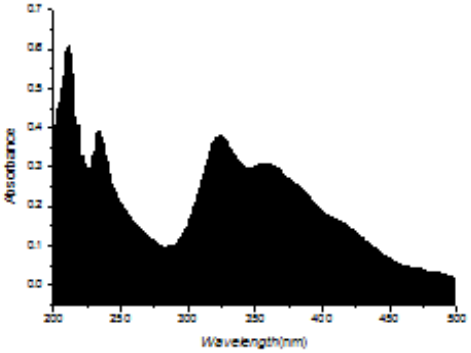
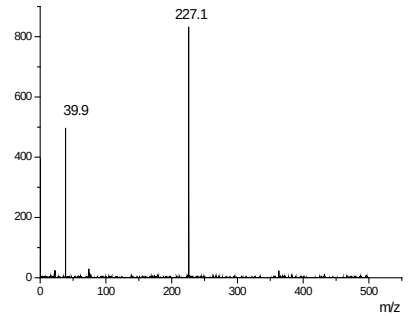
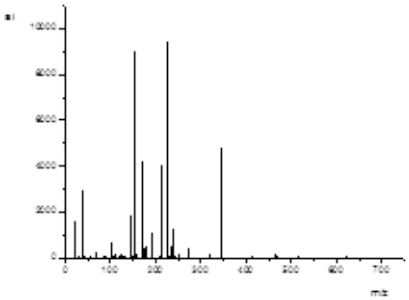


Fig. 3. 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene UV absorption diagram

As shown in Fig. 4A, the 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene matrix displays a protonated $[M + H]^+$ peak, but in Fig. 4 B, the CCA matrix shows a large number of impurity peaks within the detection area and no sample peak.



A



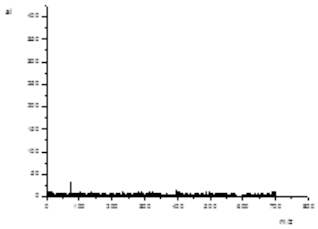
B

Fig. 4. MALDI-TOF mass spectrum of terbutaline in different matrices. A: 1,2,3,4-tetrakis (3',4'-dihydroxy phenyl) thiophene matrix; B: CCA matrix

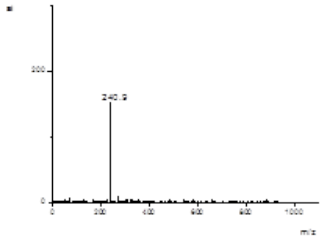
Although few sample peaks can be found in the spectum, the intensity of the sample peaks is lower and completely submerged in the impurity peaks.

Improvement of the matrix sensitivity

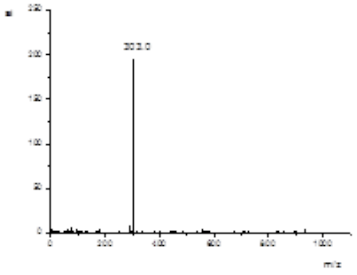
As shown in Fig. 5 A, when the sample solution is diluted to a certain concentration, sample salbutamol solution concentration is 10^{-8} mol/L under the same conditions, there is no apparent signal peak in the detection area using 1,2,3,4–tetrakis (3',4'-dihydroxy phenyl) thiophene as a matrix. Although few sample peaks can be found, the intensity of the peaks is lower and they are completely submerged in the impurity peaks. In Fig. 5 B and C, when using the mixture of 1,2,3,4–tetrakis (3',4'-dihydroxyphenyl) thiophene and perfluoro C_{60} as a matrix, the interfering peaks in the whole region are greatly reduced.



A



B



C

Fig. 5. MALDI-TOF mass spectrum for clenbuterol in different matrices. A: 1,2,3,4–tetrakis (3',4'-dihydroxy phenyl) thiophene matrix-salbutamol; B: Mixture of 1,2,3,4–tetrakis (3',4'-dihydroxyphenyl) thiophene and perfluoro C_{60} matrix-salbutamol; C: Mixture of 1,2,3,4–tetrakis (3',4'-dihydroxyphenyl) thiophene and perfluoro C_{60} matrix- ractopamine.

MALDI can determine macromolecules only; if the sample is a small molecule, a macromolecular matrix should be selected. However, a

macromolecular matrix has poor sensitivity: when the concentration of the sample solution is less than 10^{-8} mol/L it cannot produce a volume flow and significant signal peaks cannot be obtained.

In order to compensate for this deficiency, perfluoro C_{60} was added to the matrix. Perfluoro C_{60} has more hydrophobic characteristics, its structure is shown in Fig. 6. It can lock the sample in drops thus greatly increasing the sample concentration.

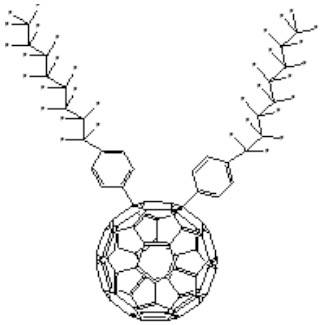


Fig. 6. Perfluoro C_{60} structure

Because of its hydrophobicity, the adsorption force of the sample at the target surface becomes weak, and the sample is easier sputtered from the target surface. Perfluoro C_{60} UV absorption diagram is shown in Fig. 7. The UV laser absorption at 355 nm can be absorbed by the sample molecules. The volume flow can be produced at lower energy. This is a good solution to the problem of poor sensitivity. Therefore, the 1,2,3,4-tetrakis (3',4'-hydroxyphenyl) thiophene mixed with the sample solution was added dropwise to the target surface uniformly covered with perfluoro C_{60} and subjected to MALDI analysis.

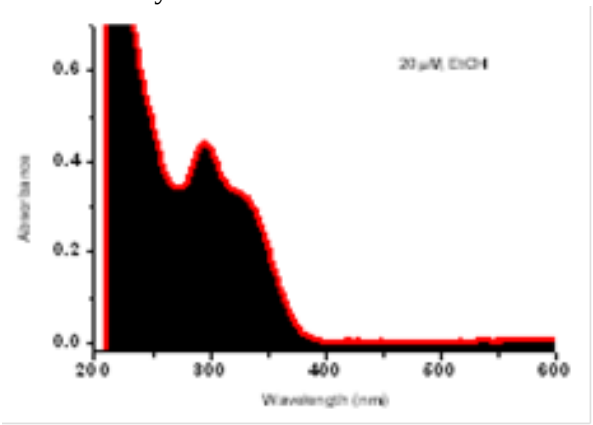


Fig. 7. Perfluoro C_{60} UV absorption diagram

When the concentration of the sample solution is less than 10^{-8} mol/L, GC-MS and LCMS cannot detect a signal in the sample and MALDI-TOF mass spectrometry should be used.

Analysis of clenbuterol

Ractopamine, salbutamol, clenbuterol and terbutaline were analyzed by MALDI-TOF MS in this experiment, the results are shown in Table 1 and Fig. 8.

In a mixture of 1,2,3,4–tetrakis (3',4'-dihydroxy phenyl) thiophene and perfluoro C_{60} as a matrix with a concentration of 10^{-3} mol/L, clenbuterol concentration in the sample solution of 10^{-2} mol/L, sodium chloride concentration of 10^{-5} mol/L and solvent: ethanol:water=1:1, MALDI-TOF MS can quickly and accurately determine the molecular weight of clenbuterol.

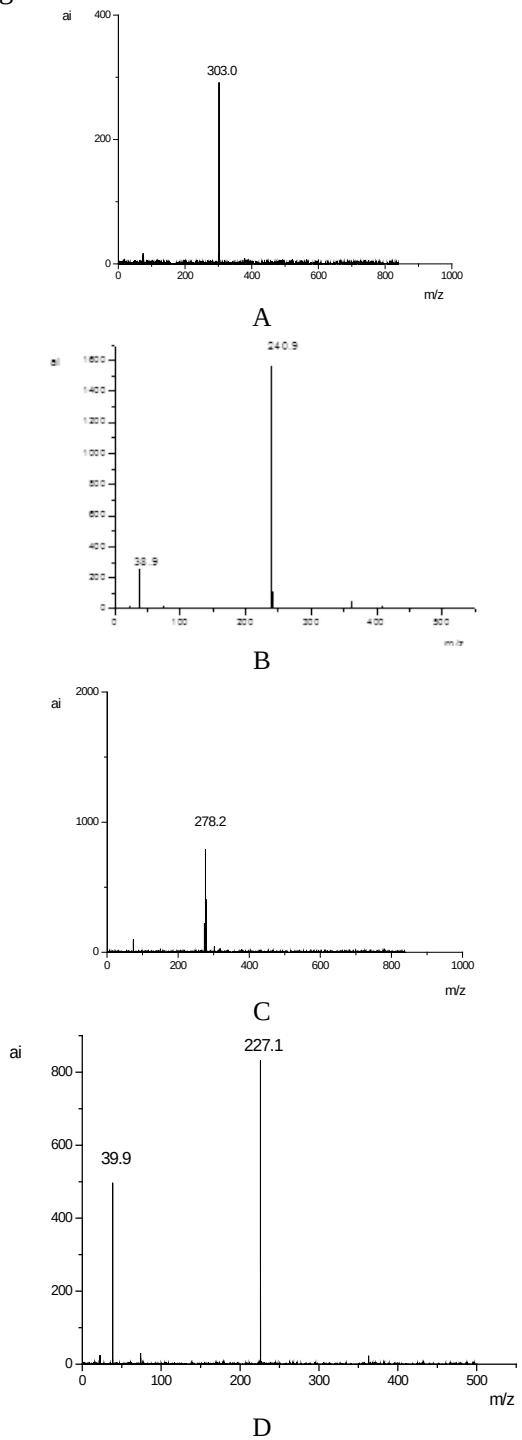
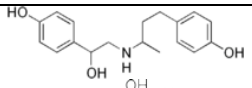
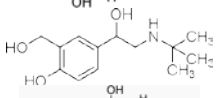
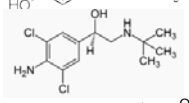
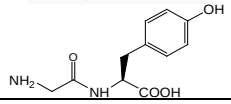


Fig. 8. The clenbuterol MALDI-TOF Mass Spectrum A: ractopamine; B: salbutamol; C: clenbuterol; D: terbutaline

Table. 1. The determination table of clenbuterol

Name	Structure	Formula	Theoretical molecular weight	Detection molecular weight
Ractopamine		$C_{18}H_{23}NO_3$	301.38	303.0
Salbutamol		$C_{13}H_{21}NO_3$	239.31	240.9
Clenbuterol		$C_{12}H_{18}Cl_2N_2O$	277.19	278.2
Terbutaline		$C_{12}H_{19}NO_3$	225.29	227.1

CONCLUSION

This paper explores the matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the small-molecule compound clenbuterol. MALDI-TOF MS has the advantages of high sensitivity, high throughput, simple operation and short analysis time. It is suitable for analysis of a variety of compounds. Using the new macromolecular matrix 1,2,3,4–tetrakis (3',4'-hydroxyphenyl) thiophene, not only a good sample can be mixed to provide a large number of protons, but also to transfer energy. Interference peaks were greatly reduced in the mass spectrum.

When the sample solution was diluted to 10^{-8} mol/L, the sample was not easily peeled off from the target surface, the macromolecular matrix had poor sensitivity, therefore, perfluoro C_{60} was introduced. Perfluoro C_{60} can lock the sample in drops of water, thereby greatly increasing sample concentration and ease of sputtering the sample from the target surface. This improved the macromolecular matrix sensitivity. The sample molecular weight was accurately measured, the error was within the allowable range, and almost no impurity peaks were present in the mass spectra. Results indicated that MALDI-TOF MS has a wide range of development and application. By taking advantage of MALDI-TOF MS high sensitivity, the presence of clenbuterol in food can be quickly and accurately detected, thus providing food security.

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Abbreviations*:* MALDI-TOF-MS: Matrix assisted laser desorption ionization time-of-flight mass spectrometry, CCA: α-cyano-4-hydroxy cinnamic acid.

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ИДЕНТИФИЦИРАНЕ НА КЛЕНБУТЕРОЛ С ПОМОЩТА НА MALDI-TOF МАССПЕКТРОМЕТРИЯ

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(Резюме)

Матрично-асистирана лазерна десорбционна йонизационна времепролитаща масспектрометрия (MALDI-TOF-MS) е използвана за анализ на макромолекулни съединения. Настоящото изследване се фокусира върху използването на MALDI за определяне на малки молекули като кленбутерол и използване на матрицата за повишаване на чувствителността. Макромолекулната матрица на 1,2,3,4-тетракис (3',4'-хидроксифенил) тиофен не само абсорбира лазерната енергия, но също води до десорбционна йонизация на пробата. В същото време тя не дава нискомолекулни пречещи йони. Добавянето на перфлуоро C_{60} към матрицата води до повишаване на чувствителността.