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Focusing Review

Development of the High Sensitive Separation Analysis Method of Metabolites in Human Nail and its Application to the Diagnosis of Chronic Disease

Jun Zhe MIN*[1,2]

[1]*Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan*

[2]*College of Pharmacy, Yanbian University, Yanji City 133002, Jilin Province, People's Republic of China*

Abstract

This review summarizes the possibility to diagnosing the chronic diseases and application of determination of metabolites of the noninvasive human nails. A target derivatization UPLC-ESI-MS method is a powerful tool for the analysis of bioactive compounds with high sensitivity and selectivity. We developed target derivatization UPLC-ESI-MS methods to analyze the biological trace components in human fingernail. Moreover, we have used these methods to detect the metabolites in human fingernails, such as DL-amino acids, polyamines and Advanced Glycation End products (AGEs). Through detecting the biological trace components in the real samples from human, we tested the possibility to use these new methods to diagnosing the chronic diseases, i.e., diabetes, lung cancer. Some applications utilizing these target derivatization methods for the analyses determination of metabolites in the human fingernails are also described in this paper.

Keywords: Human nail; Target derivatization; Diabetes; Lung cancer; UPLC-ESI-MS

1. Introduction

A target derivatization UPLC-ESI-MS method is a powerful tool for the analysis of bioactive compounds with high sensitivity and selectivity. In recent years, we have developed target derivatization methods, for determination of metabolites of the noninvasive human nails. Tissue homogenates, urine and plasma samples have been extensively investigated for the metabolites assay in biological specimens [1-3]. The inherent problems of plasma, urine and tissue homogenates, such as the fluctuation in its composition during the day, and analysis should be considered. Hygienic practice during its collection and handling is also another consideration. In contrast, the human nail is relatively clean and the samples can be quickly and noninvasively collected and easily stored. Analyzing the components of nail provides an important means for determining the individual past history of long-term chemical exposures, because many substances have been detected in the nail [4-8]. Due to the stability of drugs in nails, many studies concerning nail analysis have dealt with drugs of abuse, such as cocaine, itraconazole and amphetamines [9,10]. In the last decade, interest in nail analysis has gradually shifted to other drug species, e.g., doping agents and therapeutic drugs. According to recent reports, human nails may be used to obtain physiologic information, and may serve as a noninvasive bio sample for the diagnosis of chronic disease. Certain kinds of endogenous biogenic amino acids, polyamines and AGEs have been detected in the human nail [4-8].

However, the analysis of the target molecule is very difficult due to no fluorescence (FL) and no effective absorption in the UV-vis region. Therefore, derivatization using a suitable labeling reagent is a key step in the LC-MS analysis of the metabolites of human nails [7,8,11-16]. To date, many labeling reagents have been developed for each reactive functional group. A labeling reaction is commonly

*Corresponding author: Jun Zhe MIN
Tel: +81-54-264-5655; Fax: +81-54-264-5655
E-mail: junzhe@u-shizuoka-ken.ac.jp

achieved by the selective reaction between the functional group of the analyte and that of the labeling reagent. Labeling serves to improve the sensitivity, selectivity and the separation behavior. In our previous studies, 4-fluoro-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-F) [17], 4-(3-isothiocyanatopyrroli -din-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS) was one of the most powerful fluorescent labeling reagents [18,19]. The fluorescence reagents were applied to the sensitive determination of biological amines, such as α-lipoic acid in animal tissues, and histamine and polyamines in hair. Furthermore, the chiral derivatization reagents, e.g., DBD-PyNCS, Same applications utilizing these reagents for the analyses of bioactive chiral compounds are also described in this paper.

The aim of this review is to overview our current target derivatization UPLC-ESI-MS methods for determination of metabolites and related compounds (e.g. DL-amino acids, polyamines and AGEs in human fingernails. Furthermore, their advanced applications for usefulness of the human fingernail as a new noninvasive biological sample for the diagnosis of chronic diseases are described.

2. Determination of the intermediates of AGEs in human fingernail

The 3-Deoxyglucosone (3-DG), methylglyoxal (MG) and glyoxal (GO) possessing a reactive dicarbonyl group is an important intermediate in the formation of AGEs (Fig. 1). The AGEs are particularly important in diabetes since they have been correlated with the development of diabetic complications. Patients with diabetes have a higher concentration of amadori products because their formation is directly related to the concentration of glucose. The present study was undertaken to develop a reliable and sensitive method for the absolute quantitation of the dicarbonyl intermediates of AGEs in human nail. Preliminary testing indicated that the detection of 3-DG, MG and GO was difficult as the substance was unstable and existed in a very minute amount in human nail. A higher sensitivity is essential in any assay to detect the dicarbonyl intermediates of AGEs. Also to develop a reliable determination method to measure the free dicarbonyl intermediates of AGEs in human fingernails by UPLC-ESI-TOF-MS.

The dicarbonyl intermediates of AGEs are hydrophilic compounds. Therefore, the simultaneous separation of the dicarbonyl intermediates of AGEs by reversed-phase chromatography using an ODS column is very difficult due to the adsorption on the stationary phase compounds. In a conventional study, the 3-deoxyglucosone in rat and human plasma samples were successfully determined by HPLC separation and FL, diode array detector (DAD) [15,20]. However, these derivatization reagents have a long derivatization time, and many admixture peaks. The FL labeling is usually recommended for the determination of a real sample due to its high sensitivity and selectivity.

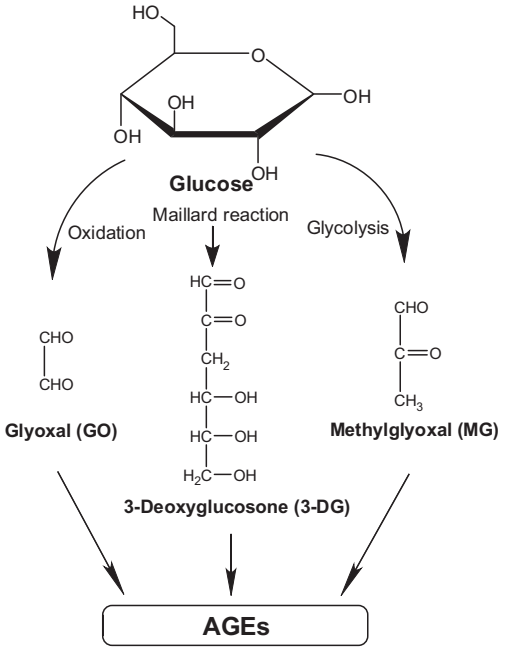

Fig. 1. Pathway of the AGEs formation.

However, the determination in complex matrices, such as hair and nails, seems to be fairly difficult by the FL detection. Indeed, the determination of several dicarbonyl intermediates of AGEs in human nails by the FL detection was interfered by any of the peaks based upon the endogenous substances. Although it seemed to be evitable through the optimization of the elution conditions, the determination within a short run time failed. Furthermore, no structural information can be obtained from the FL detection. Among the various types of available MS instruments, ESI-TOF-MS is recommended for the selective determination of target compounds because of its excellent accuracy and the precision of the resulting m/z values. Thus, the simultaneous determination of dicarbonyl intermediates of AGEs by UPLC-ESI-TOF-MS was attempted in this study. Several MS labeling reagents, i.e., o-phenylenediamine (OPD), 2-hydrazinopyridine (HP), 2-hydrazinol-methylpyridine (HMP) and 4,5-Dimethyl-1,2-phenylenediamine (DMPD), were first used for labeling the 3 dicarbonyl intermediates of AGEs. Conventional OPD had a low reaction rate with GO, and the product was not confirmed. In addition, sensitivity in the MS of the product of 3DG and MG was only half of the peak of the DMPD derivatization product. Although these reagents were essentially usable for the labeling, only DMPD efficiently labeled all the tested dicarbonyl intermediates of AGEs. Therefore, DMPD was selected for the labeling of the dicarbonyl intermediates of AGEs in the present study. The

reaction scheme of DMPD with 3-DG as a representative dicarbonyl intermediate of AGEs is shown in Fig. 2. The MS labeling effectively proceeds in a basic medium due to the electrophilic substitution reaction of DMPD for the dicarbonyl compounds. Higher temperatures are also important for the reaction to occur. Therefore, the reaction solution was heated at 60°C. The most abundant derivatives were formed for each dicarbonyl intermediates of AGEs, when the DMPD-derivatization reactions were carried out at 60°C for 2 hr. Hence, the reaction condition (60°C for 2 hr) was selected for the MS labeling of the tested dicarbonyl intermediates of AGEs.

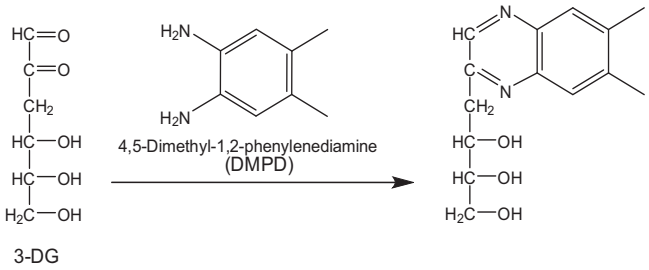

Fig. 2. Formation of the 3-DG derivative using the 4,5-dimethyl-1,2-phenylenediamine reaction.

The extracted dicarbonyl intermediates of AGEs from the fingernails of diabetic patients (age: 40-68; 10 men and 10 women) and healthy volunteers (age: 30-69; 10 men and 10 women) were then labeled with DMPD. Fig. 3 shows the typical mass chromatograms obtained from the dicarbonyl intermediates of AGEs in a fingernail from healthy volunteer and diabetic patients by the UPLC-ESI-TOF-MS. The peaks corresponding to the 3-DG, MG and GO derivatives were completely separated without any interference from the endogenous substances in the fingernails. Furthermore, a rapid separation within 7 min

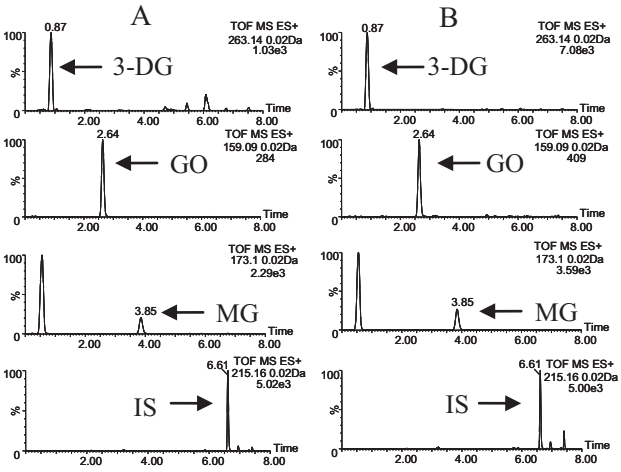

Fig. 3. Mass chromatograms obtained from the DMPD-labeled dicarbonyl compounds and the internal standard in the fingernails from healthy volunteers and the diabetic patients. A, healthy volunteers; B, diabetic patients.

was performed by the combination of the pressure-tolerant column and the UPLC instrument. Of course, the structures of the derivatives were identified from a comparison of the positive ion mode MS of the authentic dicarbonyl intermediates of AGEs. These three kinds of dicarbonyl intermediates of AGEs, i.e., 3-DG, GO and MG, were detected from the human nail samples.

Fig. 4 shows a comparison of the concentrations of the men and women's separate dicarbonyl intermediates of AGEs in the nail of healthy volunteers and the diabetic patients. When comparing the dicarbonyl intermediates of AGEs concentrations, a significant difference was observed between the 3-DG (p<0.001) in the men and women. The MG and GO were not statistically significant in the men and women. A strong correlation was observed between the 3-DG concentrations. Although the biochemical mechanisms responsible for these peculiar diabetic 3-DG profiles are unclear, the 3-DG concentration ratios might serve as a potential marker for diabetes.

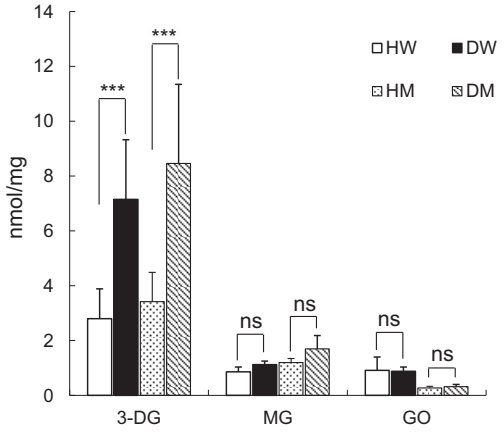

Fig. 4. Statistical analysis of the dicarbonyl compounds of men (n=10) and women (n=10) in the fingernails of healthy volunteers and the diabetic patients. HM= healthy men; DM=diabetic men; HW=healthy women; DW=diabetic women. (***p<0.001); ns: not significant.

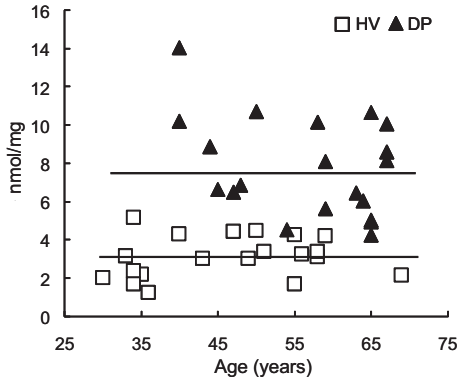

Fig. 5. Relation between the fingernail 3-DG amounts and age in the healthy volunteers and the diabetic patients. HV= healthy volunteers; DP=diabetic patients.

The 3-DG concentration was reported to increase with aging. Fig. 5 shows the concentration of 3-DG in the fingernail by the age of healthy volunteers and diabetic patients. Judging from the figure for, the concentration of the 3-DG of human nails, the concentration of the diabetic patients was higher than that of the healthy volunteers, but did not increase with age. Therefore, this analytical technique could be used as a noninvasive procedure to assist in the diagnosis and assessment of disease activity in diabetic patients.

3. Determination of DL-amino acids in human fingernail of diabetic patients

Recent studies have shown an unexpectedly wide distribution of free D-amino acids in a variety of organisms [21]. In particular, free D-Asp, D-Ser and D-Ala are found in mammalian tissues, and their physiological roles are being investigated. D-Asp is observed in various endocrine and neuroendocrine tissues and regulates the hormonal synthesis and secretion in tissues [22]. D-Ser is also localized to the frontal brain areas and regulates *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission [23-26]. In addition, D-Ala is localized in the insulin-secreting beta-cells in the pancreas, and insulin is typical regulatory hormones of blood glucose. D-Ala is suggested to have some functional relationships to blood glucose level regulation in mammals [27]. Along with the elucidation of their distributions, origins and physiological functions, the D-amino acids have been recognized as candidates of novel physiologically active substances and the marker molecules of diseases. Therefore, the high sensitive detection and accurate identification of the DL-amino acids are becoming more important for the study of their biochemical roles. However, a method for the determination of D-amino acids of the human nails has not been reported.

Fig. 6 shows the labeling reaction of amino acids with the fluorescent chiral tagging reagent, *R*(-)-DBD-PyNCS, which proceeds in a basic medium to form the corresponding diastereomers. The proposed derivatization conditions at 55°C for 20 min in aqueous acetonitrile containing 1% Triethylamine (TEA) as base catalyst were also adopted in the present research. In a previous study, the DL-amino acids in food samples were successfully determined by LC separation and FL detection [28]. However, the determination in complex matrices, such as plasma and nails, seems to be fairly difficult by the FL detection. Indeed, the determination of several DL-amino acids in human nails by the FL detection was interfered by any of the peaks based upon the endogenous substances. Although the interference seemed to be evitable by optimization of the elution conditions, the determination with a short run time failed. Among the various types of available MS instruments, ESI-TOF-MS is recommended for the selective determination of target compounds because of its excellent accuracy and the precision of the resulting m/z values. Thus, the simultaneous determination of DL-amino acids by UPLC-ESI-TOF-MS was attempted in this study. The derivatives of 17 DL-amino acids were well separated using both elution systems; i.e., the gradient elution with water-acetonitrile containing 0.1% FA, and the gradient elution with 5 mM ammonium acetate buffer 0.1% FA acetonitrile [5].

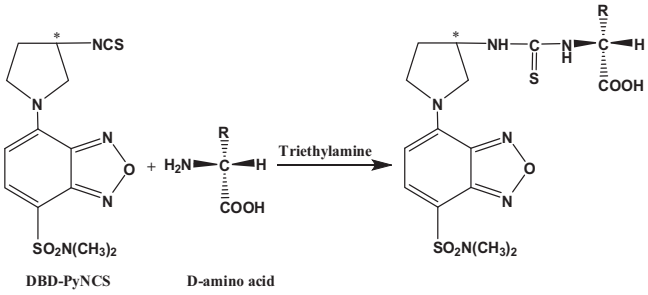

Fig. 6. Reaction of DBD-PyNCS with D-amino acids

The extracted DL-amino acids from the fingernails of diabetic patients (age: 40-64; 10 men and 10 women) and healthy volunteers (age: 33-69; 10 men and 10 women) were then labeled with *R*(-)-DBD-PyNCS. These five kinds of D-amino acids, i.e., D-Ala, D-Val, D-Pro, D-Ile and D-Leu, were detected from the human nail samples. Fifteen kinds of L-amino acids were also identified from the human fingernails. Fig. 7 shows a statistical analysis of the D/L-amino acid concentration ratios in healthy volunteers (n=20) and the diabetic patients (n=20). Significant differences (p<0.01) were observed between the Ala, Val, Ile and Leu in the healthy volunteers and diabetic patients.

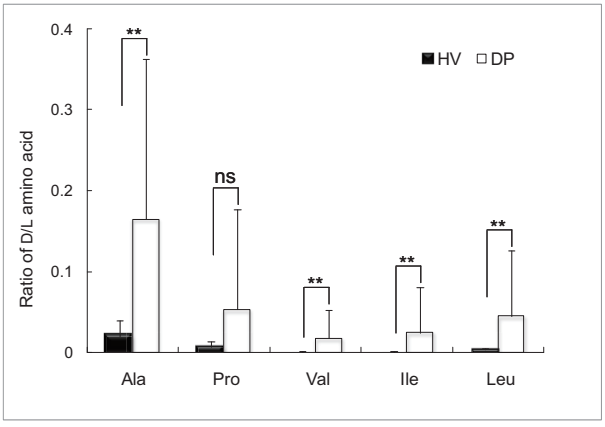

Fig. 7. Statistical analysis of D/L-amino acid ratios in the healthy volunteers (n=20) and the diabetic patients (n=20). HV= healthy volunteers; DP=diabetic patients. (** p<0.01); ns: not significant.

Furthermore, Fig. 8 shows a comparison of the concentration ratios of the men and women separate D/L-amino acid in the fingernail of healthy volunteers and the diabetic patients. When comparing the D/L-amino acid concentration ratios, significant differences were observed between the Ala (p<0.01), Val (p<0.05), Leu (p<0.05) in the men and women. Pro and Ile were statistically significant in the men, but not statistically significant in the women. A strong correlation was observed between the ratios of D/L-amino acid concentration. Although the biochemical mechanisms responsible for these peculiar diabetic D/L-amino acids profiles are unclear, the D/L-amino acid concentration ratios might serve as a potential marker for diabetes. This analytical technique could be a noninvasive procedure to assist in the diagnosis and assessment of disease activity in diabetic patients.

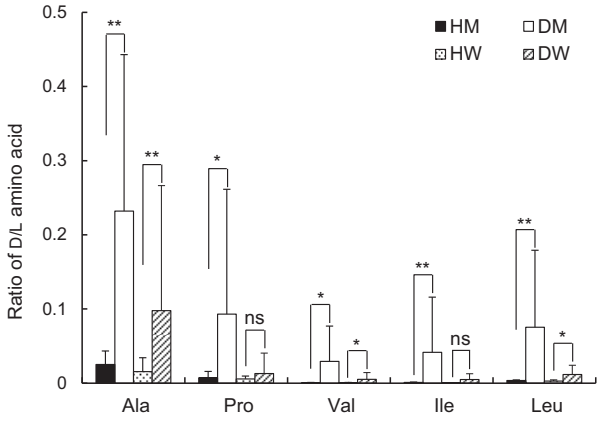

Fig. 8. Statistical analysis of D/L-amino acid ratios of men (n=10) and women (n=10) in the fingernails of healthy volunteers and the diabetic patients. HM= healthy men; DM=diabetic men; HW= healthy women; DW=diabetic women. (*p<0.05; **<0.01); ns: not significant.

4. A quantitative analysis of the polyamine in lung cancer patient fingernails

Elevated levels of polyamines have been associated with breast, colon, lung, prostate and skin cancers, and analysis of the major polyamines (putrescine, spermidine, spermine and *N*-acetylspermidine (*N*-actSPD)) and their acetyl forms in various biological specimens obtained from cancer patients has shown altered polyamine biosynthesis and accumulation [29]. Therefore the simultaneous determination of polyamines has become an important task for cancer diagnosis and antitumor drug monitoring, particularly in the study of metabonomics related to polyamines and cancer [30]. Based on these findings, the polyamines are one of the important biochemical markers for disease detection.

We developed a simultaneous determination method for polyamines in human hair and nails by UPLC-ESI-TOF-MS, nano-flow chip LC-Q-TOF-MS [4, 31]. The polyamines were labeled with DBD-F at 60 °C for 30 min in 0.1 M borax (pH 9.3). The labeling conditions required for the polyamines in human fingernails were also tested in this study. The reaction scheme of DBD-F with putrescine (PUT), used as a representative polyamine, is shown in Fig. 9. The time courses of the labeling reactions were almost complete after thirty minutes of heating. Hence, the reaction conditions of 60°C for 30 min in 0.1 M borax (pH 9.3) were selected for the subsequent DBD-F labeling of all the polyamines.

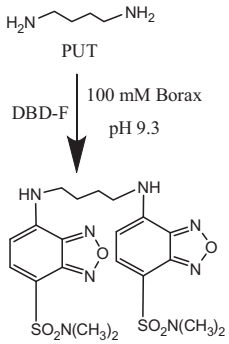

Fig. 9. Formation of the PUT derivative using the DBD-F reaction.

The extracted polyamines from the fingernails of lung cancer patients (age: 25-66; 9 men and 8 women) and healthy volunteers (age: 21-77; 19 men and 20 women) were then labeled with DBD-F. When the healthy persons were compared with the lung cancer patients, the spermine (SPM) concentrations were higher in the lung cancer patients than in the healthy volunteers. Whereas the others polyamine concentrations were similar for both the healthy volunteers and lung cancer patients. In the lung cancer patients, the others polyamine concentrations were not statistically different from those of the healthy volunteers.

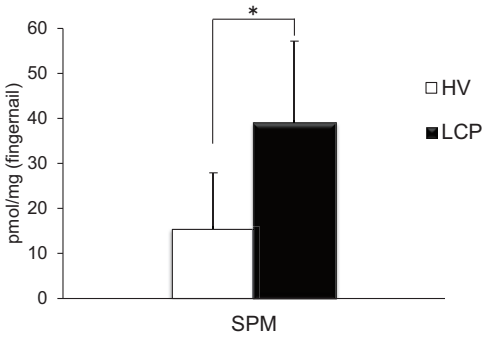

Fig. 10. Statistical analysis of the SPM in the healthy volunteers (n=39) and the lung cancer patients (n=17). HV, healthy volunteers; LCP, lung cancer patients; (* p<0.05).

Fig. 10 shows a statistical analysis of the SPM concentrations in the healthy volunteers (n=39) and the lung cancer patients (n=17). Significant differences (p<0.05) were

observed between the SPM in the healthy volunteers and lung cancer patients. A strong correlation was observed between the ratios of the SPM concentrations. Although the biochemical mechanisms responsible for these peculiar lung cancer patients' polyamine profiles are unclear, the SPM concentration ratios might serve as a potential marker for lung cancer patients. Therefore, this analytical technique could be a noninvasive procedure to assist in the diagnosis and assessment of disease activity in lung cancer patients.

5. Conclusion

We developed a target derivatization UPLC-ESI-MS method, and applied it to several real samples in order to test its cability to test the in human nails. In this paper, we described that the target derivatization methods could be powerful tools for sensitive, selective, simple and rapid determination of analytes. The proposed method was useful to detect DL-amino acids from the nails of diabetic patients and healthy volunteers. Fifteen kinds of L-amino acids were also recognized from human fingernails. There was no significant difference in the content of the L-amino acids in the fingernail. However, a statistically significant (p<0.01) and strong correlation was observed between the D/L-amino acids concentrations ratios (Ala, Val, Ile, Leu). Moreover, for the first time, identifies the importance of three kinds of dicarbonyl intermediates of AGEs in human nails. A statistically significant (p<0.001) and strong correlation was observed between the 3-DG concentrations. Furthermore, using these methods, the amounts of polyamines in the fingernails of healthy volunteers and lung cancer patients were determined. The SPM level was higher in the cancer patients. A statistically significant (p<0.05) correlation was observed between the SPM concentrations. Therefore, our findings suggest that measuring metabolites in human fingernails may be a simple, noninvasive technique to assist in the diseases diagnosis and assessment of disease activity in patients with lung cancer and diabetes. From now on, we will perform the research based on the development of the target derivatization method techniques and practically contribute to analyze biological trace components.

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