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## Short Communication

# An improved sample preparation for an LC method used in the age estimation based on aspartic acid racemization from human dentin

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The determination of age on the basis of aspartic acid (Asp) racemization in teeth is one of the most reliable and accurate methods to date. In this paper, the usefulness of HPLC coupled with fluorescence detection for determination of Asp racemization was evaluated. A modified sample preparation is proposed for better stability of *o*-phthalaldehyde-*N*-acetyl-L-cysteine derivatives of D/L-Asp (due to the instability below pH 7). To ensure the accuracy of the method, the validation parameters' specificity, precision, linearity, and LOD were determined. Three dentin samples of premolar teeth, extracted from living individuals (bucco-lingual longitudinal sections of 1 mm thickness), were analyzed and quantitative results are discussed.

**Keywords:** Age estimation / Dentin / D-Aspartic acid / Racemization / Teeth

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## 1 Introduction

In the living body, amino acids that constitute biological proteins are normally composed of the L-form, although there are some exceptional peptides which are biologically synthesized using D-form amino acids [1]. With increasing age, a gradual transformation of the L-form of amino acids in proteins into the D-forms (racemization) occurs and this racemization also occurs in metabolically inactive or bradytrophic tissues such as teeth, eye lens, vertebral discs, and parts of the brain. There are several age-related changes that occur in amino acids which constitute proteins such as oxidation, isomerization, and racemization [2, 3]. Among these changes, racemization of aspartic acid (Asp) is a first order chemical reaction [4, 5]. The determination of age on the basis of Asp racemization in teeth is one of the most reliable and accurate methods to date [6] but specialist training is necessary due to the complexity at various stages of the dentin sample preparation. This includes: collection, pulverization, demineralization, hydrolysis, derivatization,

and quantification [7]. Analytical methods so far used for the determination of L- and D-Asp include GC [6, 8–24], CE [25, 26], LC coupled with UV [27–30] and fluorescence detection [24, 31–33].

The aim of this study was to evaluate the efficiency of the LC method coupled with fluorescence detection used in the age estimation based on the determination of the D/L ratio of Asp in living human beings' dentin samples [24]. In the present paper, a modified sample preparation is proposed for better stability of *o*-phthalaldehyde-*N*-acetyl-L-cysteine (OPA-NAC) derivatives of D/L-Asp (due to the instability below pH 7). Three dentin samples isolated from premolar tooth dentin were analyzed and quantitative results are discussed.

## 2 Experimental

### 2.1 Reagents and reference substances

Reference materials of D- and L-Asp were purchased from Sigma (St. Louis, USA). *o*-Phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC) were purchased from Acros Organics (Geel, Belgium). Methanol, boric acid, hydrochloric acid, and phosphoric acid were purchased from Fischer Scientific (Loughborough, UK). Sodium azide (NaN<sub>3</sub>) was purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and sodium hydroxide (NaOH) were purchased from Riedel-de Haën (Seelze, Germany). Disodium hydrogen phosphate and disodium edetate (Na<sub>2</sub>EDTA) were purchased from Acros Organics.

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**Abbreviations:** Asp, aspartic acid; R, coefficient of racemization; NAC, *N*-acetyl-L-cysteine; OPA-NAC, *o*-phthalaldehyde-*N*-acetyl-L-cysteine; OPA, *o*-phthalaldehyde

## 2.2 Sample preparation

Three dentin samples were collected from premolar teeth, extracted from living individuals because of periodontal reasons. Bucco-lingual longitudinal sections of 1 mm thickness were prepared using a slow-speed diamond saw cutting the extracted tooth. Afterwards, the outer layers of cementum and enamel were removed using a high speed diamond bur. Both procedures were carried out under constant cooling with water in order to avoid heat related racemization of Asp. The samples of dentin were precrushed into small shards and then powdered in a vibratory mill during 15 s.

Dentin samples (5 mg) were demineralized in a 2 mL solution consisting of Na<sub>2</sub>EDTA (0.5 M, pH 7.4 adjusted with 2 M NaOH) stabilized with NaN<sub>3</sub> (0.05 mM) in centrifuge tubes. Samples were shaken intensively for 2 h, centrifuged (5000 rpm for 5 min), and the supernatant was discarded. The sediment was washed with water (2 mL each time) and centrifuged (5000 rpm for 5 min) three times to remove residues of Ca- and Mg-EDTA complexes and free EDTA. Approximately 1 mg of sediment was obtained and was transferred to a hydrolyzing tube. Hydrochloric acid (6 M, 600 µL) was added, the tubes were flame sealed and heated at 100°C for 6 h. After hydrolysis, samples were evaporated to dryness at 70°C under vacuum. All samples were prepared in duplicate.

## 2.3 Derivatization

Amino acids for LC analysis were derivatized according to the procedure previously described in the literature [24, 32, 33]. Prior to derivatization each hydrolyzate sample (approximately 0.5 mg of amino acid mixture) was dissolved in 1 mL of 0.1 M hydrochloric acid and diluted to 10.0 mL with the same solvent. A 100 µL of sample solution was mixed with 200 µL OPA-NAC reagent and left for 5 min. Then 200 µL of 0.3 M sodium phosphate buffer pH 7.5 was added and, after the mixture had stood for 5 min, 225 µL of the solution was injected into the LC system. OPA-NAC derivatization reagent was prepared by dissolving 5.5 mg of OPA in 420 µL methanol and adding 13.4 mg of NAC. The total volume of reagent was adjusted to 10 mL with 0.4 M sodium borate buffer pH 9.4. The reagent stock solution was stored at 4°C. All samples were analyzed in triplicate.

## 2.4 Instrumentation and chromatographic conditions

The LC system consisted of a Spectra SYSTEM P1000XR pump (Thermo Separations Products, San Jose, USA), an ASI-100 automated sample injector (Dionex, München, Germany), and an F-1050 Fluorescence spectrophotometer (Merck Hitachi, Darmstadt, Germany). The fluores-

cence detection was performed at  $\lambda_{\text{ex}}$  337 nm and  $\lambda_{\text{em}}$  442 nm. Data were processed by ChromPerfect® Spirit software (Justice Laboratory Software, Fife, UK). The column was kept in a water bath at 25°C. A Nova-Pak® C8 (15 cm × 3.9 mm id) 5 µm column (Waters, Milford, Massachusetts, USA) was used. The mobile phase consisted of methanol 30 mM sodium phosphate buffer, pH 5.5 (A; 10:90 v/v) (B; 90:10 v/v). The best separation between OPA-NAC-D-Asp and OPA-NAC-L-Asp was obtained using the gradient: 0–8 min (0–100% B), 8–13 min (100% B), 13–15 min (100–0% B), and 15–35 min (0% B). The flow rate was 0.5 mL/min. The injection volume was 225 µL.

## 2.5 Age determination

The following equation was used to calculate the age

coefficient of racemization (KR) =  $a \times \text{age} + b$

where KR is defined as

$$\text{KR} = \ln [1 + (D/L)/(1 - (D/L))]$$

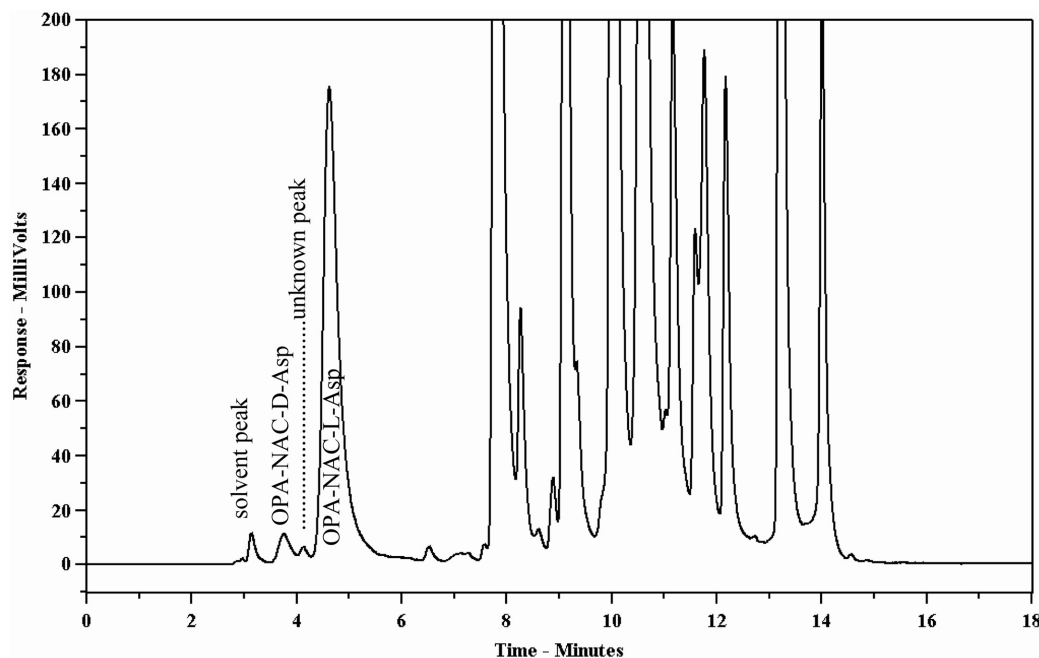
where D and L are integrated peak areas of the respective enantiomer;  $a$  represents the rate constant of racemization reaction of Asp in dentin; and  $b$  is the y-intercept.

Linear regression equation obtained by Benešová *et al.* [24] was used for the calculations.

## 3 Results and discussion

### 3.1 LC method

In this project, the LC method published by Benešová *et al.* [24] was used as a starting point. The method prescribes to use a Nova-Pak C8 5 µm column (15 cm × 3.9 mm id). The mobile phase consists of 30 mM sodium phosphate buffer, pH 5.5 (A) and methanol (B), a step gradient applied (0 min 90% A, 8 min 10% A). The separation of OPA-NAC-D-Asp and OPA-NAC-L-Asp was also examined on a 25 cm × 4.6 mm id Hypersil BDS C8 5 µm column (Thermo Hypersil-Keystone, Cheshire, UK) and a 25 cm × 4.6 mm id Nautilus C18 5 µm column (Macherey-Nagel, Düren, Germany). Both OPA-NAC-D-Asp and OPA-NAC-L-Asp were coeluted with various gradients tried. The obtained results confirm that the Nova-Pak C8 column has a special selectivity for this separation. For further experiments, the mobile phase composition was slightly adapted to have an amount of buffer in mobile phase B to avoid mixing problems and the gradient was adapted accordingly. The pH of the buffer used for sample preparation before injection was found to have significant influence on samples stability [31]. The buffer (0.3 M sodium phosphate) pH 2.0 used by Benešová *et al.* [24] was found to be inadequate for the stability of the derivatized



**Figure 1.** Typical chromatogram of derivatized amino acids obtained from human premolar tooth dentin. Chromatographic conditions: stationary phase: Nova-Pak C8 (15 cm  $\times$  3.9 mm id) 5  $\mu$ m; mobile phase: methanol-30 mM sodium phosphate buffer pH 5.5 (A; 10:90 v/v), (B; 90:10 v/v); gradient program: 0–8 min (0–100% B), 8–13 min (100% B), 13–15 min (100–0% B), and 15–35 min (0% B); flow rate: 0.5 mL/min; fluorescence detection:  $\lambda_{\text{ex}}$  337 nm and  $\lambda_{\text{em}}$  442 nm; injection volume: 225  $\mu$ L.

D/L Asp. Indeed, after 35 min at room temperature, extensive degradation of L-isomer was noted. Hence it was decided to investigate different pH values: 2.0, 4.0, and 7.5. With the 0.3 M sodium phosphate buffer pH 7.5 the D/L Asp derivatives were found to be stable for 6 h at room temperature. After 1 h at pH 4.0 and room temperature, the percentage of D/L Asp increased from 0.3 to 2.9%. Various gradient methods were performed to examine the separation between OPA-NAC-D-Asp and OPA-NAC-L-Asp. Conditions mentioned under Section 2.4 were found to be the best. A system peak always eluted with the OPA-NAC-L-Asp. The area of this system peak is subtracted from the area of OPA-NAC-L-Asp before the calculation. A typical chromatogram of dentin is shown in Fig. 1.

## 3.2 Quantitative aspects

### 3.2.1 Identification

The retention times of OPA-NAC-D-Asp (3.8 min) and OPA-NAC-L-Asp (4.7 min) in dentin samples corresponded to those of derivatized reference materials of D- and L-Asp.

### 3.2.2 Precision

The repeatability is given by the RSD calculated on the peak area ratio of D- and L-Asp. It was calculated from six determinations of the standard solution, consisting of

6 ng of D-Asp and 600 ng of L-Asp *per* mL. The RSD found was 2.7%.

### 3.2.3 Linearity

Linearity of D/L Asp ratios at constant concentration of L-Asp was measured in the expected range of D-Asp concentration, *i.e.*, from 6 to 60 ng/mL of D-Asp (1–10%) with a constant concentration of the L-form of 600 ng/mL (100%, calculated according to the average amount of Asp in hydrolyzate). The D/L ratio of the peak areas was linear in the measured range (Table 1).

### 3.2.4 LOD

LOD is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOD was assessed by injecting OPA-NAC-D-Asp at various concentrations, the amount of OPA-NAC-L-Asp (100% = 600 ng/mL) was always kept constant. The LOD, corresponding to an S/N of 3, obtained for OPA-NAC-D-Asp is 3 ng/mL (0.5%).

## 3.3 Age determination

Three dental samples of known age (two samplings from 70 years, one sampling each from 13 and 15 years) were analyzed for the determination of the KR of Asp and the results obtained are summarized in Table 2.

**Table 1.** The linearity of D/L Asp area ratios at constant concentration of L-Asp in the expected range of D-Asp concentrations

D-Asp injected range	Regression equation $y$	$r^2$	$S_{y,x}$	$n_c$	$n_i$
1.0–10% (6–60 ng/mL)	$0.0079x + 0.0017$	0.9989	0.0017	3	3

$y$ : Ratio of area obtained for OPA-NAC-D-Asp to the area of OPA-NAC-L-Asp;  $x$ : % of OPA-NAC-D-Asp to OPA-NAC-L-Asp (m/m);  $r^2$ : coefficient of determination;  $S_{y,x}$ : standard error of  $y$ -estimate;  $n_c$ : number of experimental concentrations studied;  $n_i$ : number of injections for each concentration.

**Table 2.** Estimation of known age dentin samples based on coefficients of racemization (KR) of Asp

Actual age (years)	Sample no.	D/L-Asp ratio	KR	%RSD ( $n = 3$ )	Estimated age (years) <sup>a)</sup>
70	1.1	0.036871	0.073776	1.2	95
	1.2	0.043503	0.087061	1.4	122
	2.1	0.044714	0.096175	0.3	140
	2.2	0.046931	0.093931	1.4	135
	Mean	0.043005	0.087736		123
13	3.1	0.017806	0.035615	1.8	19
	3.2	0.017943	0.035889	1.9	19
	Mean	0.017875	0.035752		19
15	4.1	0.018165	0.036333	4.1	20
	4.2	0.018004	0.036012	0.1	20
	Mean	0.018085	0.036173		20

$n$ : Number of experiments.

<sup>a)</sup> The estimated ages were calculated by regression equation  $KR = 0.0005 \times \text{age} + 0.0262$  [24].

Each sample of known age was prepared several times and analyzed three times (see Table 2). The RSD values of KR found were in the range 0.3% (sample of 70 years age) to 4.0% (sample of 15 years age). The ages were calculated using the regression equation  $KR = 0.0005 \times \text{age} + 0.0262$  [24] and the results are summarized in Table 2. All samples were overestimated with respect to the known age due to the variability of test tooth and dentin. This shows that it is necessary to prepare a regression equation in each laboratory intending to apply the technique.

## 4 Concluding remarks

An improved sample preparation was proposed for better stability of OPA-NAC derivatives of D/L-Asp. In combination with the proposed sample preparation, the LC method is linear and repeatable and the method can be used for the determination of age based on the racemization of Asp in dentin. However, for the accurate estimation of age, it is necessary to study each time the test tooth along with four or more control teeth of known ages, which are homonymous teeth from the same jaw as the test tooth [34].

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