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USE OF ASPARTIC ACID RACEMIZATION IN FINGERPRINTS AS A MOLECULAR CLOCK

Work submitted by

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for obtaining the Master's Degree in Forensic Science

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Dedicated to my parents, María Gracia and Manuel.

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Certificate of originality

This is to certify that I am responsible for the work submitted in this thesis and that the work is original and not copied or plagiarized from any other source, except as specified in the acknowledgements and in references. Neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

Signature:

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Date: 15th July 2014

Abstract

Aspartic acid is the chemical, amongst amino acids, whose racemization process is considered to be the fastest and the most reliable way to conduct an ageing study in some living-being tissues. Racemization is a natural-occurring event in which L-stereoisomer of aspartic acid is converted into D-stereoisomer in an equilibrium dependent on factors namely pH, environmental conditions and, most critically, on temperature.

The main objective of the current study is to assess the influence of temperature on kinetics of aspartic acid racemization and to implement this methodology in fingermarks. The hypothesis is that different starting points of deposition of a biological specimen will give rise to significant differences in L-/D- racemization after the sample has been heated, allowing to determine time since deposition of a fingerprint. In this way, it would be possible to find the time since build a molecular clock for fingerprints, consisting of time since deposition of fingermarks based on racemization degree of aspartic acid.

Firstly, a calibration curve was built within the concentrations range expected to be quantified in actual samples both with L-aspartic acid (L-Asp) and D-aspartic acid (D-Asp) separately, Gas Chromatography - Flame Ionization Detector (GC-FID) being the chosen technique and detection mode to set up the calibration experiments.

Secondly, L-aspartic acid standards were treated at different temperatures during different time periods and were analyzed by GC-FID with a chiral column, aimed for detecting D- isomer, providing with information about racemization rate of aspartic acid.

Eventually a downward trend was observed for L-Asp but not for D-Asp and a pseudo-rate constant could be calculated for the former, demonstrating that temperature degrades this chemical. Fingerprints study was not successful to determine aspartic acid racemization but a simple handling of them and an adequate, sensitive response of the chromatographic system could quantify racemization degree.

Keywords: *aspartic acid, isomers, racemization, fingerprints, time, deposition, clock, influence, temperature*

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Abbreviations list

AAR: Aspartic Acid Racemization

BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide

CAS: Chemical Abstracts Service

CE: Capillary Electrophoresis

D-Asp: D-Aspartic acid

ECF: Ethyl Chloroformate

FID: Flame Ionization Detector

GC: Gas Chromatography

HCl: Hydrochloric acid

HMW: Heavy Molecular Weight proteins

HP-5: (5% Phenyl)-methylpolysiloxane

HPLC: High-Performance Liquid Chromatography

$\mathbf{k_{Asp}}$: rate constant for aspartic acid racemization

L-Asp: L-Aspartic acid

MS: Mass Spectrometry

PA-ACS: analytical grade reagents which meet specific requirements from American Chemical Society

RPE: R-Phycoerythrin

TLC: Thin-Layer Chromatography

WI: Water-Insoluble proteins

Contents

1. Introduction

1.1. Fingerprints composition

Fingermarks chemical composition involves a wide range of chemicals and determining them all is not an easy task since fingerprints come from different sources, not only sweat as can be thought (Girod *et al.*, 2012). The five sources in which fingermarks substances can be present are: sebum secretions, apocrine sweat, eccrine sweat, epidermic substances and external environmental conditions (Weyermann *et al.*, 2011).

To determine fingermarks compounds two main aspects must be considered: composition right after deposition and aged composition, that is, degradation of their chemicals as a function of time. It has also been proven that, after deposition of fingerprints onto a surface, there are several factors that affect their composition, namely donor, substrate of deposition, techniques utilized for enhancement and environmental conditions.

Age is one of the main aspects involved in composition change as children and adults do not house the same chemicals on their fingertips. (Antoine *et al*., 2010). It has been proven that fingerprints between younger and older individuals can be clearly distinguished because cholesterol, cholesteryl esters and long-chain fatty acids are abundant in children fingertips whilst adults show a very low amount of them, hence the first conclusion is that a decrease of these compounds is produced over time.

Pressure is another variable that affect chemical composition of fingerprints and vacuum conditions lead to a change in them in relation to ambient pressure (Bright *et al.*, 2013). It was estimated that fingerprint samples subject to vacuum suffer a loss of more than one fourth of their fat content, which can be compared to a five-week decomposition process at normal pressure. Nonetheless, this loss has only been assessed in lipids, fatty acids, their corresponding esters and water.

Light and temperature are amongst environmental factors that promote a degradation of compounds such as squalene and cholesterol, both of them being completely degraded in 15 days time at room conditions (Amorós *et al.*, 2014) but squalene might not be detected in some cases when 9 days are passed, having stored it under light conditions (Archer *et al.*, 2005).

1.2. Analytical determination of fingerprints components

Typically, chromatography is the most utilized technique to analyze chemicals present in fingerprints and several studies have been conducted to show the advantages and drawbacks of the different chromatographic modalities.

Gas Chromatography - Mass Spectrometry (GC-MS) is the desirable technique due to its extremely low sensitivity, its high resolution and its confirmatory power. On these lines, an innovative study by Croxton *et al*. (2006) introduced this combination and results provided with thirty-minute chromatograms in which 12 amino acids were fully identified as well as free fatty acids and all peaks belonging to them were separated with acceptable resolution.

MS might also be coupled to Capillary Electrophoresis (CE) and it could constitute an excellent alternative when no chromatographic equipment is available. Amino acids are prone to be analyzed by CE due to their zwitterionic nature and many studies have been conducted over last decade about amino acid determination in diverse kinds of samples with fluorescence and MS as main detection techniques (for a review see Poinsot *et al.*, 2010). CE-MS has been proven to be fit-for-purpose in the case of amino acids analysis in fingerprints with detection and quantitation limits in the region of ng (Atherton *et al.*, 2012).

However, High-Performance Liquid Chromatography (HPLC) has been proven to work in a suitable way and it can also be useful to detect certain kind of disease like beta-thalassemia (Khedr, 2010). A potential problem that HPLC shows is its time-consuming feature, which leads to long chromatograms, hence net time analysis becomes a drawback but a shorter time would promote coelution of some amino acids. HPLC can also be employed to seek degradation products of squalene, providing MS is the detection technique or even Thin-Layer Chromatography (TLC) could be useful for screening if there is a previous knowledge about the compounds (Mountfort *et al.*, 2007).

1.3. Aspartic acid: chemistry and isomers

Aspartic acid is one of the 20 amino acids contained in proteins, classified into the group of amino acids with a negatively charged R group due to its carboxyl group in its lateral chain, which is ionized when pH exceeds its pK_R (3.65) (Nelson and Cox, 2004). Like all amino acids (with the exception of glycine), aspartic acid has one stereogenic center which is the reason of its two stereoisomers: L- and D-, the former being the one present in living organisms and subject to racemization during lifetime in a natural-occurring process.

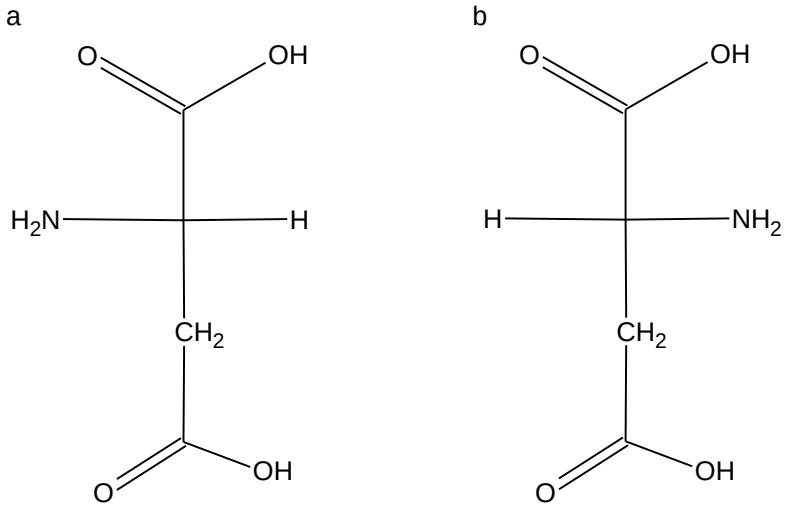


Figure 1: Chemical composition and functional groups of L- (a) and D- (b) stereoisomers of aspartic acid.

D-aspartic acid (D-Asp), despite not being contained into proteins, is normally used as an enhancer of testosterone levels when taken as a supplement in any diet, especially for athletes to improve their performance, though it is not demonstrated either its efficacy or its health benefits (Ładyga and Obmiński, 2013).

1.4. Aspartic acid racemization (AAR) process

One peculiarity of this chemical is its reliability as an age marker when its racemization over time is taking place in every kind of tissues, which has led it to become an excellent compound to monitor when conducting an ageing process. According to a study performed by Arany and Ohtani (2010), aspartic acid seems to be the only appropriate to carry out this sort of experiments as other amino acids namely glutamate and alanine did not show results as accurate as aspartic acid provided, even when considering those amino acids from the soluble peptide fraction. Standard error for aspartic acid seems to be the lowest possible, making studies more precise when determining age. The reason behind this fact is that aspartic acid is the one more prone to suffer racemization, the rest being much slower in this particular sense.

A literature review by Zapico and Ubelaker (2013), summarizes chemical methods to simulate an ageing process with many types of tissues (namely dentin, cementum, white matter of brain or cartilage) performed by several authors in several studies, in which AAR is one of those methods. Another viewpoint is given by Ritz-Timme and Collins (2002), in which a good understanding can be gained about the importance of AAR as a chemical process occurring in proteins and as an effective age marker for proteins.

The reaction of racemization and isomerization is well explained and summarized by Cloos and Fledelius in a study conducted in 2000. The mechanism of the reaction proceeds as follows: a nucleophilic attack takes place by the action of the nitrogen atom of the peptide bond on the carbonyl on its side and a succinimide ring, which is very unstable, is formed. This succinimide is sensitive to hydrolysis and racemization, hence is degraded to peptides in D- and L- forms, producing the native peptide and its racemized, isomerized and both modifications together in an equilibrium at any time.

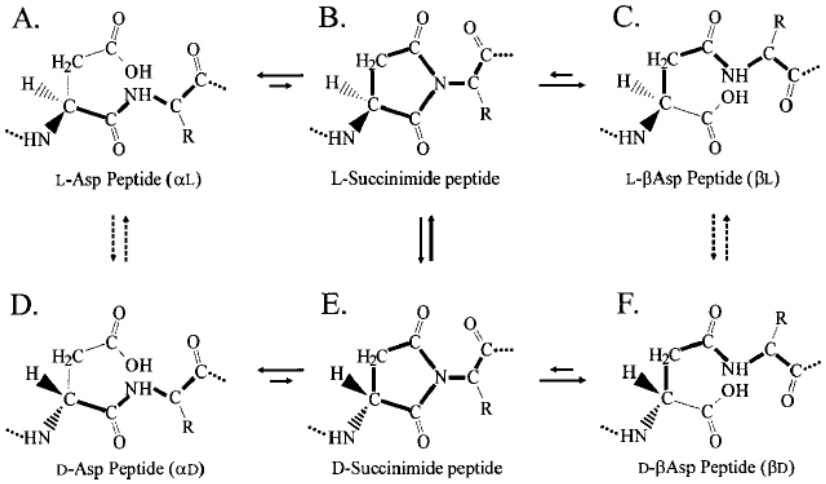


Figure 2: Chemical route of racemization followed by aspartic acid with all equilibriums taking place between stereoisomers and conformations (taken from Cloos and Fledelius, 2000).

In 1958, Kuhn already mentioned a possible correlation between the interconversion of L- and D- stereoisomers of amino acids and the aging process, and several attempts have been recorded over decades as this is an issue that has appealed the interest of scientists since the seventies regarding forensic purposes.

Bada and Protsch (1973) introduced AAR as a suitable alternative to radiocarbon analysis to determine age of fossil bones providing that temperature is the same both for older and younger specimens. Aspartic acid had been demonstrated to show a racemization half-life of 15,000-20,000 years at 20 ºC and even other amino acids namely isoleucine and alanine were shown to racemize at a slower rate (100,000-150,000 years) at 20 ºC.

In 1975, Helfman and Bada already established the first chemical method to determine age based directly on amount of D-Asp in teeth produced over time. An increase in this chemical was observed with age of the enamel and this could be correlated to actual age of the individual whereas the same did not occur in hemoglobin as D-Asp remains constant with human age. This breakthrough was essential in this topic and, thanks to this discovery, other authors have reported how to determine AAR attempting with other kind of tissues or even the same. They even achieved to quantify the amount of D-

Asp once 60 years were passed and it was found that 8% of aspartic acid had been racemized to D- stereoisomer. Bada and Schroeder (1975) provided with kinetic aspects about AAR, this being a reversible, first-order reaction depending mostly on pH and temperature.

1.5. AAR monitoring in tissues – analytical determination

Since the seventies, many studies have been conducted to assess AAR in every kind of tissues in living beings and a chronological review can provide with a better understanding about breakthroughs and steps given forward in this field.

Masters *et al*. (1978) found AAR in human lenses and cataracts both in heavy molecular weight (HMW) and water-insoluble (WI) proteins from these tissues and it was found that D-Asp is directly related to formation of WI, which could be the previous step after aggregation of HMW.

Ritz *et al*. (1990) developed a method to assess AAR in dentin which presented the advantage of reproducibility, useful for further studies, and it was shown that no specialization in terms of knowledge and skills is needed when conducting an aging study based on AAR. Besides, influence of temperature was not considered as it is not supposed to change in the body of an individual but research should be intended to monitor any likely temperature change, specially regarding weather conditions.

Ritz and Schütz (1993) were dealing with intervertebral discs to support studies that had already established age of death from dental tissue and they provided a very useful aid by analyzing nucleus pulposus and annulus fibrosus. Racemization process was shown to be much faster in the former than in the latter because of the environment in which these tissues are located, which can favour or unfavour it. Anyway, this study was not as accurate as a research conducted with dentin, which had already been proven to be the most effective at that point.

Typically, aspartic acid is determined by chromatography, mostly GC and HPLC and the former being predominant, and there are many studies dealing with these modalities. Mörnstad *et al*. (1994) had already proven that reversed phase HPLC could become a suitable technique to quantify D-/L- ratio given a very high reliability of results that could made it comparable to GC in relative terms, taking into account that the latter is costlier even though provides better resolution and sensitivity high enough can be achieved with both. On these lines, Yekkala *et al*. (2006) developed a fluorescence detection-based HPLC method which was proven to provide with excellent results in terms of D-/L- ratios and correlation coefficients and a more recent study conducted by Garde *et al*. in 2010 also showed a high correlation between AAR and age, hence HPLC has indeed become an alternative. Sivan *et al*. (2012) were also utilizing it in order to test racemization process in elastin from both normal and degenerate tissue and D-Asp was found to accumulate to a greater extent in the former rather than in the latter.

As every chemical reaction, AAR is affected by environmental factors and one of them is indeed pH. Ohtani (1995) verified how pH can increase or decrease racemization rate and it was demonstrated, in the light of results, that temperature is a more critical factor that makes the reaction speed up as long as the former increases and there is also a dependence on pH which leads racemization to go faster as the environment is more basic, that is, when pH is higher. This happened to occur only in teeth since bones act as a buffer against pH changes and this study could not demonstrate the reason why an alkaline medium favours racemization, attributing it to differences in collagen composition.

An interesting study conducted by Carolan *et al*. (1997) showed why aspartic acid is the most reliable amino acid, when compared to glutamic acid and serine, to estimate age even from ancient bones like the ones presented in this article. The main reason behind is the faster racemization degree compared not only to glutamic acid and serine but to the rest of amino acids.

Quality assurance is indeed an essential aspect to pay attention at and Ritz-Timme *et al*. (2000), from that concern, attempted to suggest an unified criteria in terms of personal skills and training, experimental design to foresee likely changes in the outcome and detailed guidelines are given when developing the methodology, from sample collection and handling to data treatment. Interlaboratory and intralaboratory assessments are also encouraged but, as this study concludes, these are general guidelines and any institution or laboratory has to apply them to their daily routine according to their necessities.

Ritz-Timme and Collins (2003) proposed an innovative, novel methodology to assess AAR in elastin from human skin, which supposed a promising step forward to determine this parameter in other tissue different from dentin, which had been the most used over last decades. Racemization process happened to occur more slowly than expected but the reason behind this event was the biochemical environment since collagen structure contains tertiary conformations which slow down the formation of succinimide, the limitant step in AAR. It was also demonstrated, in the light of results, that temperature is a critical factor when monitoring racemization as an increase of temperature denatures proteins, making structural complexity of them not a handicap any more for the process, therefore it evolves faster and it is not representative for a living organism. This is a fact also demonstrated in a research by Dobberstein *et al*. (2008). Hence temperature needs to be controlled every single time when conducting any kind of these experiments.

Arany *et al*. (2004) implemented internal standard methodology to GC analysis and this was as advantageous as expected since the use of an internal standard permits to circumvent unavoidable errors in sample handling and injection. However, not any chemical could serve as a good internal standard and this study demonstrated that D-methionine provides better results than D-norleucine in terms of chromatographic resolution and linearity of the calibration curve, hence the former is recommended for these purposes.

Ohtani *et al*. (2005) wanted to avoid the step of collecting dentin samples from individuals and they decided to attempt the same analyses normally performed on tooth but only with mixtures of aspartic acid standards and a previous knowledge of racemization in dental tissue. Results obtained were as reliable as the ones from a normal study in real tooth samples, hence this would be a good manner to conduct racemization monitoring when unknown samples are no available and there is a need of research.

Alveolar bone was the tissue selected by Ohtani *et al*. (2007) to study AAR and to separate both enantiomers in a very short period of time (only five minutes) by gas chromatography with a special column. However, the main limitation of this research was the difficulty of performing these analyses in women as the correlation for them was very low unlike in men, in which the method was proven to work appropriately for estimation of age.

Fernández *et al*. (2009) linked AAR to DNA recovery but to a very limited extent since it was only possible with endogeneous strains. However, this small discovery happened to be an excellent approach and further research should be conducted in order to correlate racemization and age of old remains. On these lines, Poinar *et al*. (1996) had already been working on this topic and they could only recover DNA from samples with a very low racemization degree, as well as in the previous case, hence no reliable correlation could be found between DNA and amino acid racemization. Collins *et al*. (2009) were also attempting to find any link as kinetics of AAR is thought to be similar to DNA depurination and they provided a reason for this fact, likely to happen because of the biochemical environment in which collagen is involved. Its *quaternary* helix seemed to be behind a very limited racemization extent which makes impossible to find this relationship between AAR and DNA recovery. Offele *et al*. (2007) demonstrated that AAR can reveal changes in molecular DNA and maceration is highlighted as a sample handling that may reduce the reliability of AAR determination due to additional racemization introduced by this.

Griffin *et al*. (2010) assessed the degree of AAR in enamel from different sorts of tooth as it had been shown that this extent was dissimilar in the mere enamel when analyzing dentin from other individuals. This study was not able to explain that variation as acid soluble fraction from enamel provided results which were not as precise as the ones obtained by Ohtani *et al*. (2005), in a research that was focused on an only individual with different teeth. Key factors of variations in AAR is the time of formation of the particular tooth and temperature since teeth more exposed to the environment will suffer a lower racemization process than the one located deeper, where temperature will be higher.

Thorpe *et al*. (2010) linked D-aspartic acid accumulation to horses' collagen and non-collagenous proteins contained in two sorts of tendons and they established half-life of those proteins according to the content of D-Asp. It was also demonstrated how temperature affects AAR in tooth it is normally 33 ºC and, in tendon, is 37 ºC, which makes constant rate double according to calculations performed.

Alkass *et al*. (2010) introduced for the first time a combination of AAR and radiocarbon analysis to conduct an ageing process and results showed that both techniques did correlate and disadvantages of the former can be outweighed or masked by advantages of the latter. Hence it is possible to determine age of birth and age of death by merging results from both techniques though radiocarbon analysis shows better results as regards precision and accuracy, leaving a promising future for this methodology in coming years.

Ohtani and Yamamoto (2011) decided to assess influence of ethnic groups on racemization rate in teeth but this study failed to obtain a correlation between them since there were so significant differences in racemization across the diverse origins studied but intrinsic properties of teeth played a more decisive role and this was the key factor to confirm the failure of this experiment.

Besides, a series of experiments with bear dentine remains in Spanish caves were developed by Torres (de) *et al*. (2001 and 2002) and a reliable correlation between AAR and date of this kind of remains, caves being an appropriate place to sample since its environment is not subject to contamination and hence there is no decrease in racemization apart from the good, stable thermal conditions inside.

Arany and Ohtani (2011) achieved to perform AAR analysis in bloodstains, which had never been attempted at that point because the main tissues monitored had been tooth and bone, mainly. They obtained a kinetic model to predict racemization bearing in mind that this is a first-order chemical reaction (already demonstrated) and influence of temperature was studied, leading to excellent correlation results for statistical analyses. A novel, reliable methodology was pursued and achieved in this research.

Sakuma *et al*. (2012) were establishing a comparison between dentin tissue and whole-tooth samples to evaluate in which of them racemization was more noticeable and they found out that the latter shows a higher racemization degree, associating it with the presence of cementum, tooth pulp and enamel (not in dentine) but on the other hand correlation was weaker in whole tooth because of the excellent result in dentine. In spite of everything, both results were statistically significant.

Matzenauer *et al*. (2013) proposed an alternative when dentine samples, which are the most reliable to determine AAR as an age marker, are no longer available. Epiglottis samples were selected and experiments were carried out with elastin tissue both purified and taken from cadavers during autopsy and results proved that this particular tissue is a feasible option for monitoring AAR, although dentine keeps being the most appropriate kind of tissue to gather the most accurate, reliable results that can be linked to age.

Rajkumari *et al*. (2013) decided to conduct a study in order to assess the suitability of HPLC as an analysis technique to determine AAR in dentine tissue, already tested as the best sort of samples for this purpose. It was demonstrated that it is an alternative to GC and can provide results which permit to match racemization with age of an individual and it is remarkable to bear in mind that this process is not affected by gender and jaws in different teeth.

Onstott *et al*. (2014) have recently given a step forward to attempt to monitor AAR in planktonic organisms from South Africa, showing that they are indeed living organisms since their D/L ratio was very slow and protein turnover and replacement is still ongoing in that kind of cells. Very recently, *Torres et al*. (2014) have also presented a review about AAR in mammals from historical and prehistorical dates. The most important and factor was demonstrated to be sample handling, faster and more effective than in other methods already tested for aging studies such as electron spin resonance or ^{14}C.

1.6. Influence of temperature on AAR process

It has been thoroughly demonstrated that temperature is a critical factor on AAR and an increase in the former leads to an increase in the latter. Therefore, rate constant for racemization (k_{asp}) will always raise as temperature does in every kind of tissue. There are several studies showing that AAR speeds up as temperature increases and, as explained in section 1.5, racemization is enhanced when both a high tempreature and a high pH are applied (Ohtani, 1995). Luzanna *et al*. (1999) found an increase of 8 times in k_{Asp} when temperature raised from 95 ºC to 120 ºC in fish material. Thorpe *et al*. (2010) showed in collagen that k_{Asp} is multiplied by a factor of 2 when temperature suffers an increment of 4 ºC. In bloodstains, when temperature is increased from 90 ºC to 120 ºC k_{Asp} raises by 31 times (Arany and Ohtani, 2011).

1.7. Derivatization prior to GC analysis

Chloroformates have been utilized as successful derivatizing reagents in many cases, as stated in a review by Schurig (2011), due to their many advantages: reaction happening at room temperature, ease of method automation, not expensive and no large amounts of organic solvents employed. However, sometimes a by-product is formed depending on the alcohol used as a medium and combination of alcohol with chloroformate (Abe *et al*., 1996). This can be easily solved if the alkyl of the alcohol is more electronegative than the alkyl of the chloroformate. Another example of this effectiveness is given by Namera *et al*. (2002). Hušek (1998) summarized in a very explanatory way how chloroformates were employed in amine treatment and they became effective derivatizing reagents for amino acids analyses as well. This same author, in 1995, had already begun using ethyl chloroformate in a very first approach to this kind of organic solvents. Recently propyl chloroformate has been tested as an efficient solvent to recover up to nine amino acids in fingerprints with correlation coefficients higher than 0.96, which means an acceptable reproducibility although the rest could not be detected (Mink *et al.*, 2013).

The reaction taking place between ethyl chloroformate and an amino acid is summarized in this way.

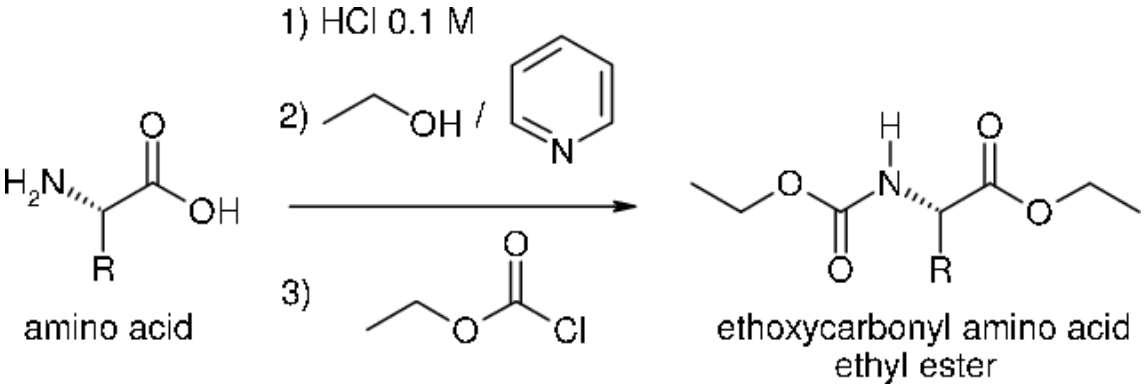


Figure 3: General reaction of an amino acid with ECF to obtain the corresponding volatile ester, in this way subject to analysis by gas chromatography (taken from Nuevo *et al*., 2006).

However, Stenerson (2011) showed that silylation is a good alternative in spite of sensitivity levels achieved, not as low as it could be expected. Another drawback, as seen later, is high temperature needed for the reaction to occur, which can lead to sample losses. This is the general reaction taking place for carboxyl and amine group with trimethylsilyl reagents.

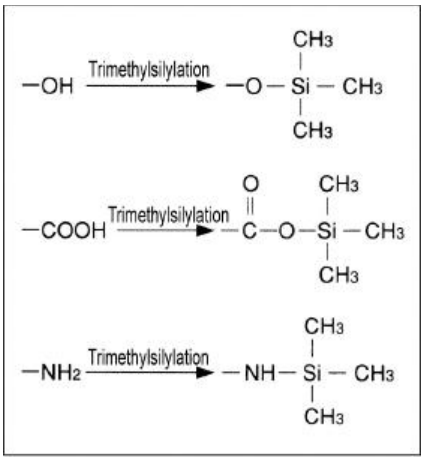


Figure 4: General scheme of trimethylsilyl reagents reaction with some functional groups, producing volatile substituted compounds to be analyzed by gas chromatography (applicable to aspartic acid) (taken from http://www.wako chem.co.jp/english/labchem/product/analytical/chromato_v8/a45/index.htm).

1.8. Aims and objectives

This research is intended to implement a novel methodology for AAR determination in forensic evidence namely fingerprints and, for this reason, a key factor such as influence of temperature on AAR will be assessed with several experiments at different exposure times and different temperatures. Furthermore, AAR has been proven to be the most reliable and fast when being racemized. Fingerprints have an unequivocal feature when linking this evidence to a person thus, in this way, it would be possible to determine time passed since the fingerprint was deposited onto a surface according to racemization extent. For this purpose, samples to be collected will go across different genders, ages and ethnicities, as well as considerations regarding diet, medication or cosmetics employed by every single participant. Sample preparation will be intended to denature proteins present in fingerprints with the aim to enhance the presence of aspartic acid and a subsequent derivatization to make it volatile. Hence it will be subject to a GC-FID analysis with a chiral column to separate its L and D stereoisomers.

2. Materials and methods

2.1. Reagents and solutions

All reagents are shown next together with purity and CAS number in brackets. Hydrochloric acid (37%, 7647-01-0) was obtained from Panreac (Barcelona - Spain), as well as pyridine PA-ACS (110-86-1). L-Asp (reagent grade ≥ 98%, 56-84-8), D-Asp (99%, 51186-58-4) and ethyl chloroformate (≥ 98% (GC), 541-41-3) were obtained from Sigma Aldrich (France, South Korea and Hungary, respectively). DL-norleucine (616-06-8) was supplied by Sigma Chemical CO (St Louis, USA) and BSTFA (99%, 25561-30-2) was obtained from Supelco (Bellefonte, USA). Carlo Erba group was in charge of providing chloroform RPE (67-66-3) from Rodano (Italy) and acetonitrile HPLC-plus gradient (200-835-2), from Chaussée du Vexin (Val de Reuil, France).

A 1 mg/mL stock solution of L-Asp and D-Asp were prepared in water separately and, from this one, three more diluted solutions of 15 µg/mL, 50 µg/mL and 100 µg/mL, all of them in hydrochloric acid (HCl) 0.1 M. Internal standard methodology was chosen for this research and the chemical acting as a internal standard was DL-norleucine, of which a solution 1 mg/mL in water was prepared.

2.2. Derivatization strategies

2.2.1. BSTFA

A methodology adapted from Stenerson (2011) was implemented. 50 µL of L-Asp in HCl 0.1 M from each of the three concentrations available were placed into a 2-mL glass react vial. Internal standard (DL-norleucine) was added in a fixed amount to all the samples, hence 3 µL of DL-norleucine solution 1 mg/mL were placed. Both L-Asp and DL-norleucine were dried under nitrogen stream and, once this dryness process was finished, 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (derivatization reagent) and 100 µL of acetonitrile were added. This mixture was heated at 100ºC during 4 hours into a water bath and eventually 1 µL was injected into the GC-FID system.

2.2.2. ECF

A methodology previously developed by Croxton *et al*. (2010) was adapted for this research. Three replicates for each of the three concentration levels together with a blank were prepared, hence 10 samples were analyzed by GC-FID. For every single calibration standard, 60 µL of the L-Asp in HCl 0.1 M were placed into a tube at each concentration, for the blank being only HCl 0.1 M with no L-Asp. 3 µL of the internal standard solution (DL-norleucine 1 mg/mL) were added to every single tube followed by a mixture of absolute ethanol, pyridine and ethyl chloroformate (ECF) 32:8:5 (v/v/v), manual stirring after adding the latter and 100 µL of chloroform (containing 1% ECF) were added to this mixture. Two phases were clearly observed after samples vortexed. 1 µL of the organic phase was collected and injected into the GC-FID system.

2.3. Determination by Gas Chromatography - Flame Ionization Detector (GC-FID)

An Agilent 7820A GC System was employed to conduct all analyses. This device is coupled to a Flame Ionization Detector and used a HP-5 column (325 ºC: 30 m x 320 µm x 0.25 µm) for standards and quantifying purposes and a chiral column Chirasil L-Val (200 ºC: 25 m x 250 µm x 0.12 µm) for stereisomers separation and ratio between them. Injection was manual with 1 µL of sample. Programme temperature in the oven was set up as follows: 60 ºC during 1 min, 35 ºC/min up to 290 ºC, holding 3 min and 40 ºC/min up to 300 ºC, with a total duration of 10.89 min for each chromatogram and splitless mode in the case of standards and calibration curve. For separation of stereoisomers the programme was amended as shown: isotherm at 60 ºC during 3 min to ensure a complete solvent elution, 20 ºC/min up to 140 ºC, holding 3 min on top, total duration being 10 min for each chromatogram and split 1:5 in this modality.

Injector temperature and detector temperature were 250 ºC and 350 ºC respectively. Gases flow through the detector were in all cases 30 mL/min for hydrogen, 400 mL/min for air and 28 mL/min for nitrogen, used as a makeup. All chromatograms were acquired and treated with the software GC 7820A.

2.4. Method optimization

Method to be followed for the rest of the research was selected amongst BSTFA and ECF as derivatization reagents. Eventually the protocol was the same explained in section 2.2.2. However, agitation happened to be a critical factor and it was necessary to optimize reaction time with ECF. For this reason, different analyses were performed in such a way to allow the reaction to occur and three replicates at 0, 10, 20, 30, 40, 50 and 60 min after adding ECF were prepared, all of them at room temperature, only for 100 µg/mL of L-Asp. Reaction times were measured while stirring automatically at 600 rpm. Once these times were passed, 100 µL of chloroform containing 1% ECF were added and 1 µL of the organic phase (at the bottom of the tube) was injected into the GC-FID.

2.5. Calibration curve obtaining

To obtain every single calibrator, the procedure mentioned in section 2.4 was followed, once reaction time with ECF was fixed (explained in section 3.1.2). Concentrations were chosen to be in the range of 30-180 µg/mL.

2.6. Study of influence of temperature on racemization reaction

Once the calibration curve was obtained, next step was assessing how temperature is able to speed up the racemization process. For this purpose, three different temperature values were chosen: 112 ºC, 116 ºC and 120 ºC to expose several L-Asp and D-Asp standards during different times at those temperatures. Times selected were: between 2 hours and 2 days with equidistant points in between. The protocol followed was: a high concentration of L-Asp (1 mg/mL) to ensure an adequate response in the chromatographic system was selected and three replicates were used for each time at each temperature. 200 µL of L-Asp (1 mg/mL) were taken from the initial stock solution and then placed into a 1.5-mL Eppendorf tube. Various thermoblocks (Bioblock Scientific - USA and Labnet International - USA) were employed to heat all the samples at the different temperatures established.

2.7. Fingerprints collection

Beforehand, an approval was obtained from the ethical commission of this institution and nineteen donors were selected according to key factors such as gender, age and ethnicity. They were requested not to wash their hands one hour prior to collection in order to avoid lipids losses and hence any change on fingertips composition. They all had previously signed an informed consent form and all samples and information about donors were treated as anonymous, assigning a number to each donor. They were asked about age, ethnicity, diet followed, smoking habits, medication taken at that point and cosmetic employed on the collection day. Fingerprints were collected onto a sheet 2 cm x 2 cm in the following way: donors were asked to print once with each of their fingertips on the same surface in order to ensure a proper collection. Samples were collected during a whole evening and were kept individually in envelopes to treat them the following day.

Table 1: Information about participants in the study, separated by gender and taking into account information such as age, ethnicity, diet, smoking habits, medication taken currently and cosmetics applied on the collection day.

Gender		Age		Ethnicity		Diet		Smoking		Medication		Cosmetics	
Male	9	20-29	14	White	10	Omnivore	17	Y	2	Y	6	Y	7
Female	10	30-65	5	Black	5	Vegetarian (no meat)	2	N	17	N	13	N	12
				Middle Eastern	1								
				South Asian	1								
				Central / South American	2								

2.8. Fingerprint samples handling

The day after collection, each sheet was cut in smaller pieces and introduced in an Eppendorf tube, in which 500 µL of HCl 6 M were added and every sample was heated at 80 ºC during 24 h. Afterwards, all the liquid content inside each tube was transferred to a new one, all tubes were opened, a bit of parafilm was attached on top and some holes were made by poking the parafilm. Tubes were frozen at -72 ºC during 2 h and, once this time was passed, they were placed into a lyophilizer to stay at vaccum pressure and -50 ºC overnight. The objective of this sample treatment was to denature proteins present in fingerprints and to enhance the presence of free amino acids, which is of interest to detect the presence of aspartic acid.

2.9. Statistical analyses

Microsoft Excel 2010 was utilized to plot all the charts and bar graphs shown in the next section.

3. Results and discussion

3.1. Aspartic acid derivatization

3.1.1. Non-suitability of BSTFA-based methodology

It was noticeable that this procedure was not suitable for the purpose initially established since samples suffered losses partial or total losses. In principle, the reason could be the high temperature applied (100 ºC). In this sense, only 100 µg/mL of L-Asp were employed for assays where temperature was decreased down to 50 ºC and diverse experiences were conducted at different reaction times: 30, 90, 150 and 210 min, three replicates for each reaction time, showing similar problems later than 90 min, when samples began to evaporate. The advantage of this kind of samples is that they can be kept at very low temperatures with no damage in their composition, hence they were kept in a freezer since the GC-FID detector was not working properly in that particular day and they were injected the day after. Nonetheless, though not having complete vaporization of the earlier samples, they did not show any peak in the chromatograms, hence this was an indication that the reaction was not happening. This is a typical chromatogram when this methodology was followed.

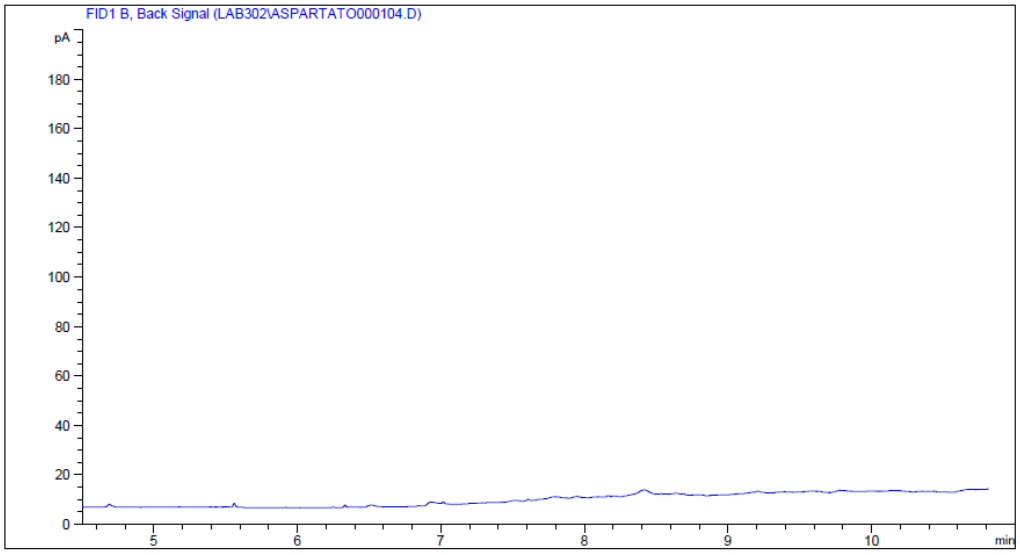


Figure 5: General profile of a chromatogram obtained when analyzing a derivatized L-Asp standard with BSTFA by GC-FID with a HP-5 column.

Temperature was increased to 60 ºC and the experiment was repeated only at short times (15 min and 30 min) with three replicates for each reaction time and there were no sample losses but peaks in the chromatogram were absent, therefore the reaction was not occurring at any extent or so low that it was not detectable, showing chromatograms like the previous one shown above. It is also remarkable that solvents employed in this experimental procedure (BSTFA and acetonitrile) are extremely volatile by themselves (45-55 ºC and 81-82 ºC respectively) and they can begin evaporating at the temperatures applied for these experiments. However, it is already reported in literature (Stenerson, 2011; Schummer *et al.*, 2009; Molnár-Perl and Kaltona, 2000) how this method works at high temperatures between 50 ºC up to 150 ºC and solvents are not lost because of the heat applied, so there is a further reason to explain the behaviour of the solution. React vials material might not have been appropriate for this experiment as it was expected that the mixture did not evaporate to any extent after being heated, hence another kind of vial or material should be used for this purpose.

3.1.2. Appropriate derivatization with ECF and reaction time optimization

After BSTFA assays had been inconclusive and methodology based on it was proven to be ineffective, the decision adopted was to move to ECF. The sample mixture with L-Asp and internal standard was prepared and derivatized (stated in section 2.2). First experiments showed a profusion of peaks when only two peaks (corresponding to L-Asp and (DL)-norleucine) were expected and it could have given food to believe that this derivatization reagent was not suitable for the purpose initially pursued. An example of this observation is given below.

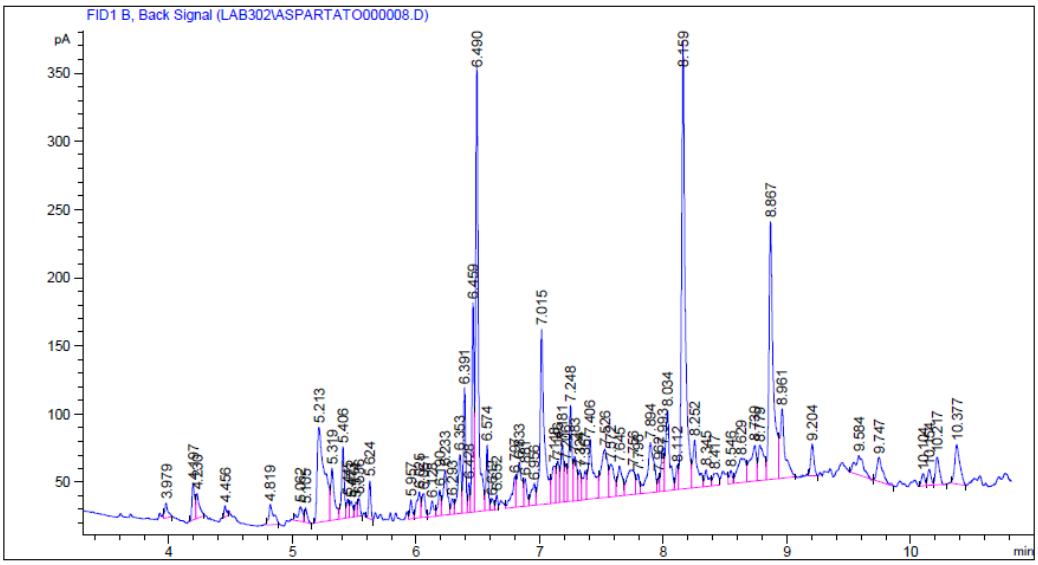


Figure 6: General profile of a chromatogram for a L-Asp standard when applying ECF as a derivatization reagent and manual agitation for a few seconds. Sample injected into a GC-FID device with a HP-5 column.

In the light of results with almost no agitation, all the peaks appearing on the chromatograms did not seem to be noise, hence their appearance could be due to an incomplete reaction of ECF with the functional groups of both L-Asp and internal standard. As shown in Figure 7, L-Asp contains three substituents prone to be derivatized (two carboxyl groups and one amino group), whilst norleucine houses two groups (one carboxyl and one amino). An incomplete reaction of ECF with these functional groups could have originated these numerous peaks and only one peak is of interest for aspartic acid and another peak for internal standard. In each case, the single peak expected should correspond to a complete derivatization of all functional groups of each chemical. Besides, injections were made with splitless mode and this modality was chosen to obtain better peaks because the purpose of this stage of the research was to quantify but, on the other hand, this promoted that all these undesirable peaks appeared and with a split modality these mentioned peaks could have been removed or minimized. A higher split ensures narrower peaks and avoids the obtention of chromatograms like the one shown above.

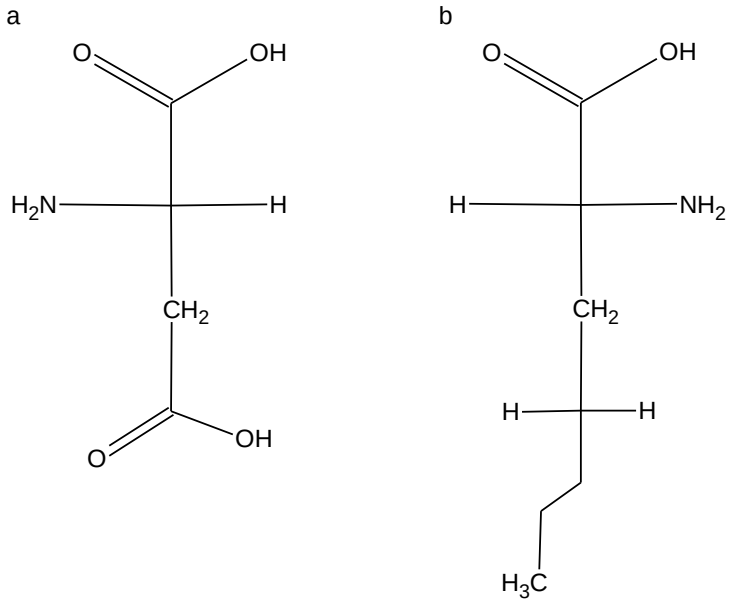


Figure 7: Chemical structure and comparative between L-Asp and D-Norleucine.

As it seemed that the derivatization reaction was incomplete, so it was suggested that the reason behind this event could be agitation after addition of ECF. Thus manual stirring was replaced by automatic, continuous agitation. Besides, reaction time might not have been long enough to allow the reaction to occur, therefore with a longer period of time the reaction could be completed. In this way, reaction time of aspartic acid and internal standard with ECF needed to be optimized. To achieve it, different samples were prepared and they were allowed to react with ECF at different times (0, 10, 20, 30, 40, 50 and 60 min), in each of which an automatic agitation was occurring. Chromatograms for each of them were acquired and peak areas for only L-Asp (to evaluate its increase) were plotted against agitation time in order to obtain a certain time in which the minimum agitation produced the maximum amount of derivatized product.

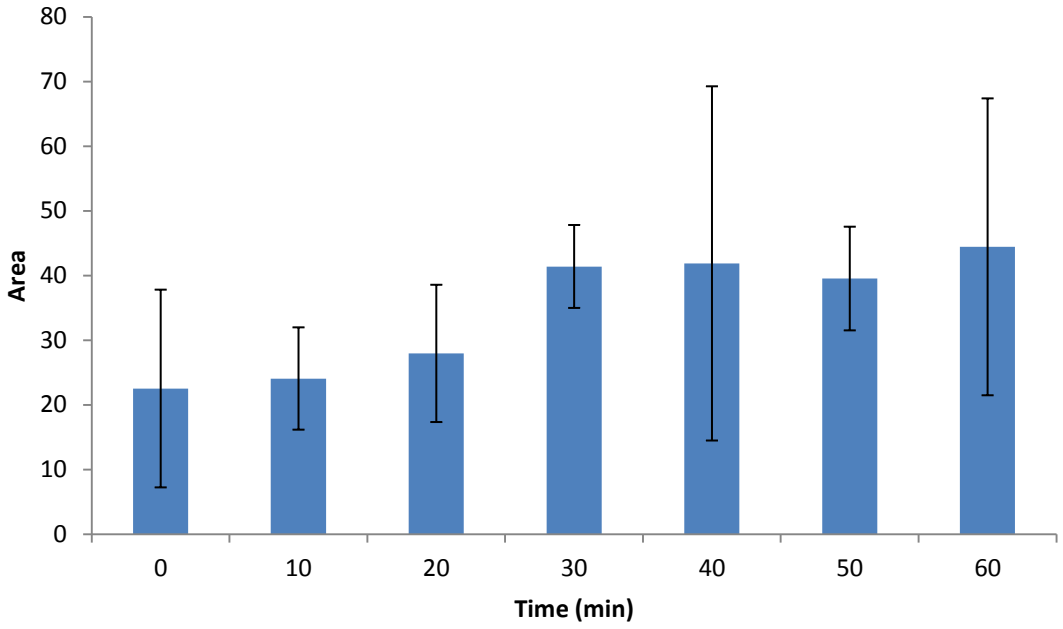


Figure 8: Bar graph plotting chromatogram peak areas of L-Asp vs. reaction time with ECF, samples being analyzed by GC-FID with a HP-5 column.

Areas represent the amount of product originated from the derivatization reaction of L-Asp with ECF and it is reasonable to believe that, from 30 minutes and further ahead, there is no great increase in derivatized amino acid and peak areas will not raise to a great extent, hence reaction time was established in 30 minutes since the reaction was thought to last long enough to produce the maximum amount desired.

When the method was optimized with 30-min reaction, chromatograms obtained showed considerably less peaks than the previous ones commented and this was the methodology followed during the rest of the research. There were still more peaks than the two expected, hence it was necessary to analyze a blank to reject the rest of the peaks, corresponding to a still incomplete derivatization reaction of aspartic acid. An example of the chromatograms profile once methodology was optimized is shown below for both L-Asp and D-Asp.

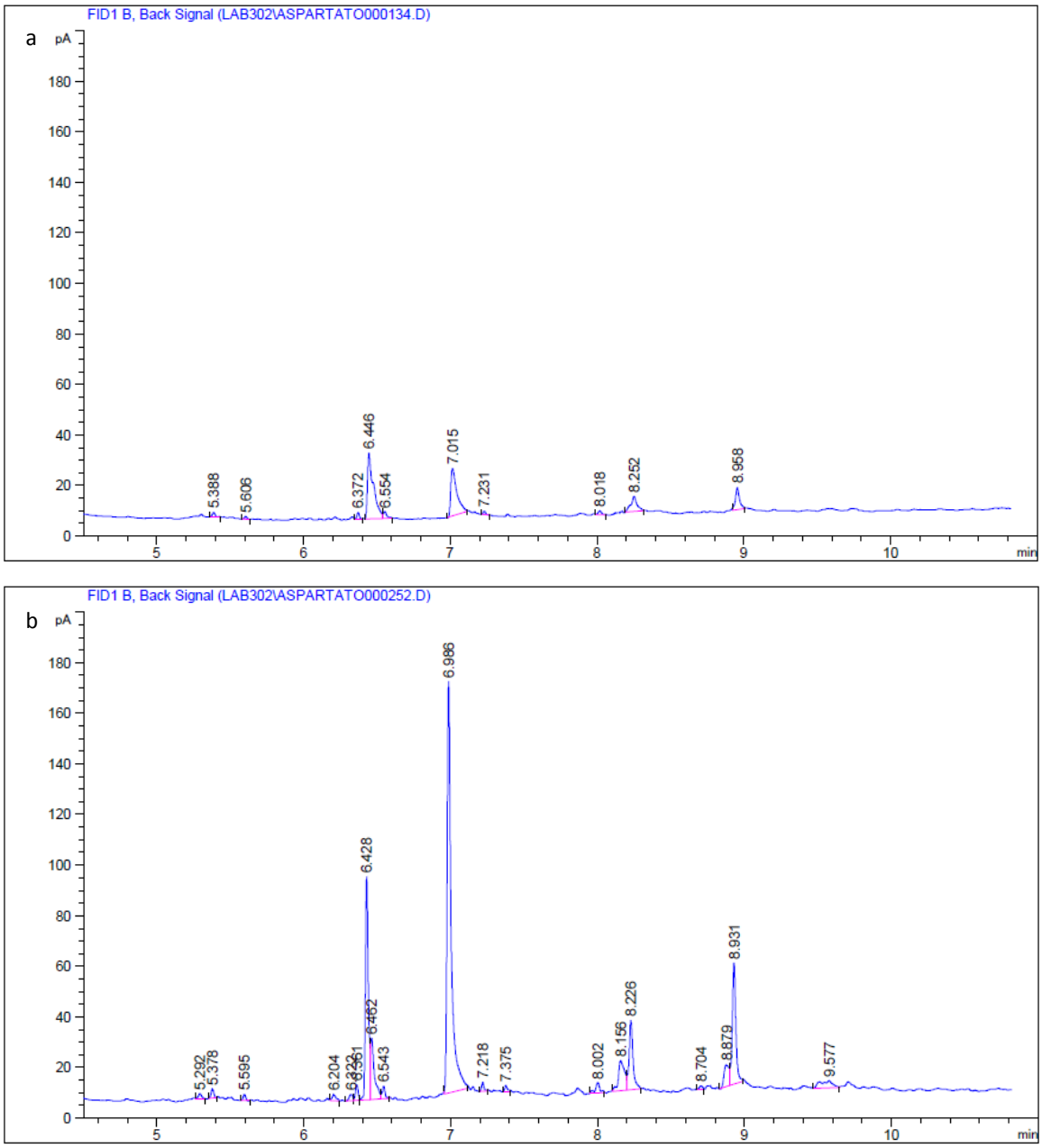


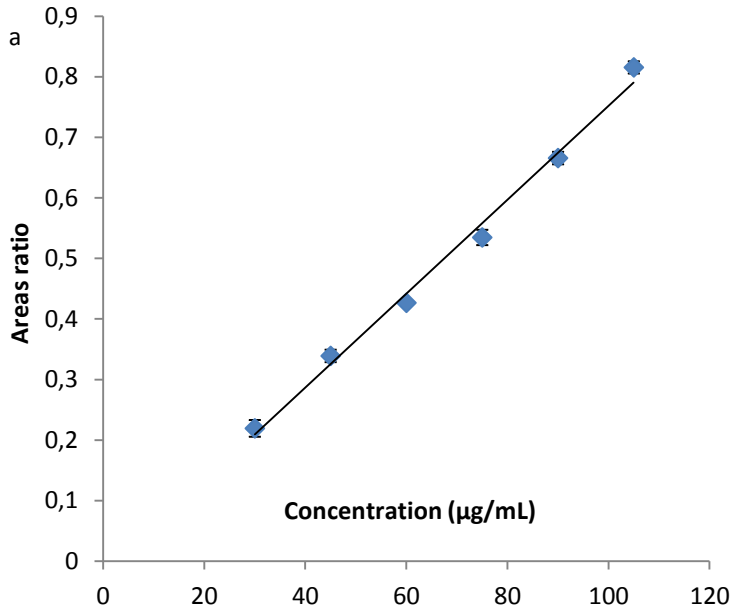
Figure 9: General profile of a chromatogram when: (a) analyzing a L-Asp and (b) analyzing a D-Asp standard by GC-FID with a HP-5 column, applying derivatization methodology with ECF. In (a) internal standard (DL-norleucine) eluted at 6.446 min and L-Asp, at 7.015 min whereas in (b) internal standard (DL-norleucine) eluted at 6.428 min and D-Asp, at 6.986 min.

3.2. Calibration results

The initial idea was to build a calibration curve with a concentration range between 30 µg/mL and 180 µg/mL. However, calibration curve obtained showed a correlation coefficient which was not accurate enough to obtain a reliable calibration. In fact, error bars obtained were very wide at higher concentrations, next to 180 µg/mL. Besides, the lowest concentration could not be lesser than 30 µg/mL because the instrument was able to quantify it and probably at lower levels it would not be possible to obtain a peak, hence the range was narrowed, starting in 30 µg/mL and each point being separated 15 µg/mL. Results are shown next for both stereoisomers together with statistical results.

Table 2: Statistical results of calibration curves for L-Asp and D-Asp, following the general equation of a straight line (y=ax+b), together with correlation coefficient.

Stereoisomer	a	b	R^2
L-Asp	0.0078	-0.0232	0.9926
D-Asp	0.0202	0.0471	0.9926



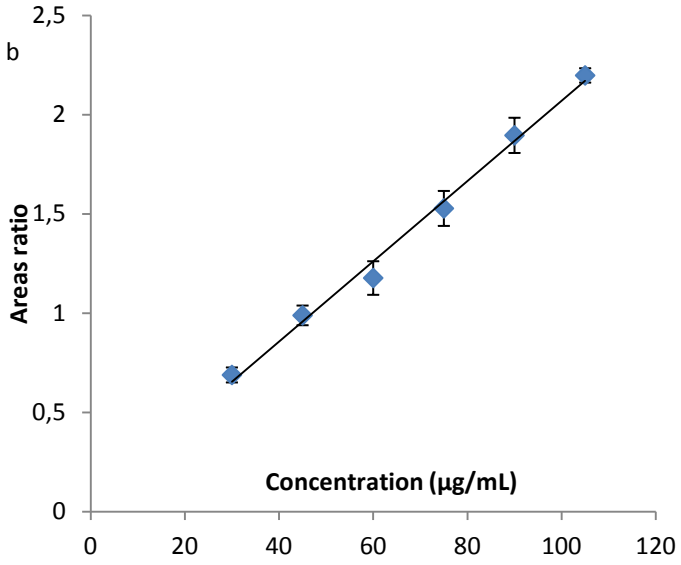


Figure 10: Calibration curves for (a) L-Asp and (b) D-Asp, both derivatized with ECF, concentration range between 30-105 µg/mL. Areas ratio (y axis) refers to quotient between area of aspartic acid into area of internal standard (DL-norleucine), obtained by injecting all the calibrators into a GC-FID system with a HP-5 column.

This calibration curve was intented to quantify L-Asp and D-Asp separately in fingerprints once both stereoisomers were identified in fingermarks but, as explained later on, these isomers could not be identified in fingerprint samples collected due to a likely failure of the chiral column aimed for separating the isomers or the non-sensitive feature of the GC-FID device utilized.

3.3. Kinetic assessment of aspartic acid racemization

3.3.1. Influence of temperature on L-Asp degradation

The main objective was to assess racemization degree of aspartic acid by obtaining the ratio between L- and D- isomers. The chiral column was used for this purpose and a split of 1:5 was selected in this case. The reason for choosing this modality and no splitless (as in the previous section) was that the two peaks expected to appear in chromatograms (D-Asp and L-Asp) were going to elute very close to each other. As

explained before, a high split guarantees narrow peaks but the split could not be very high for not losing more sample and hence the peaks expected.

Firstly, L-Asp and D-Asp, after being treated and derivatized as stated in previous sections, were injected to identify retention time of each of them and a blank to reject peaks not corresponding to both isomers.

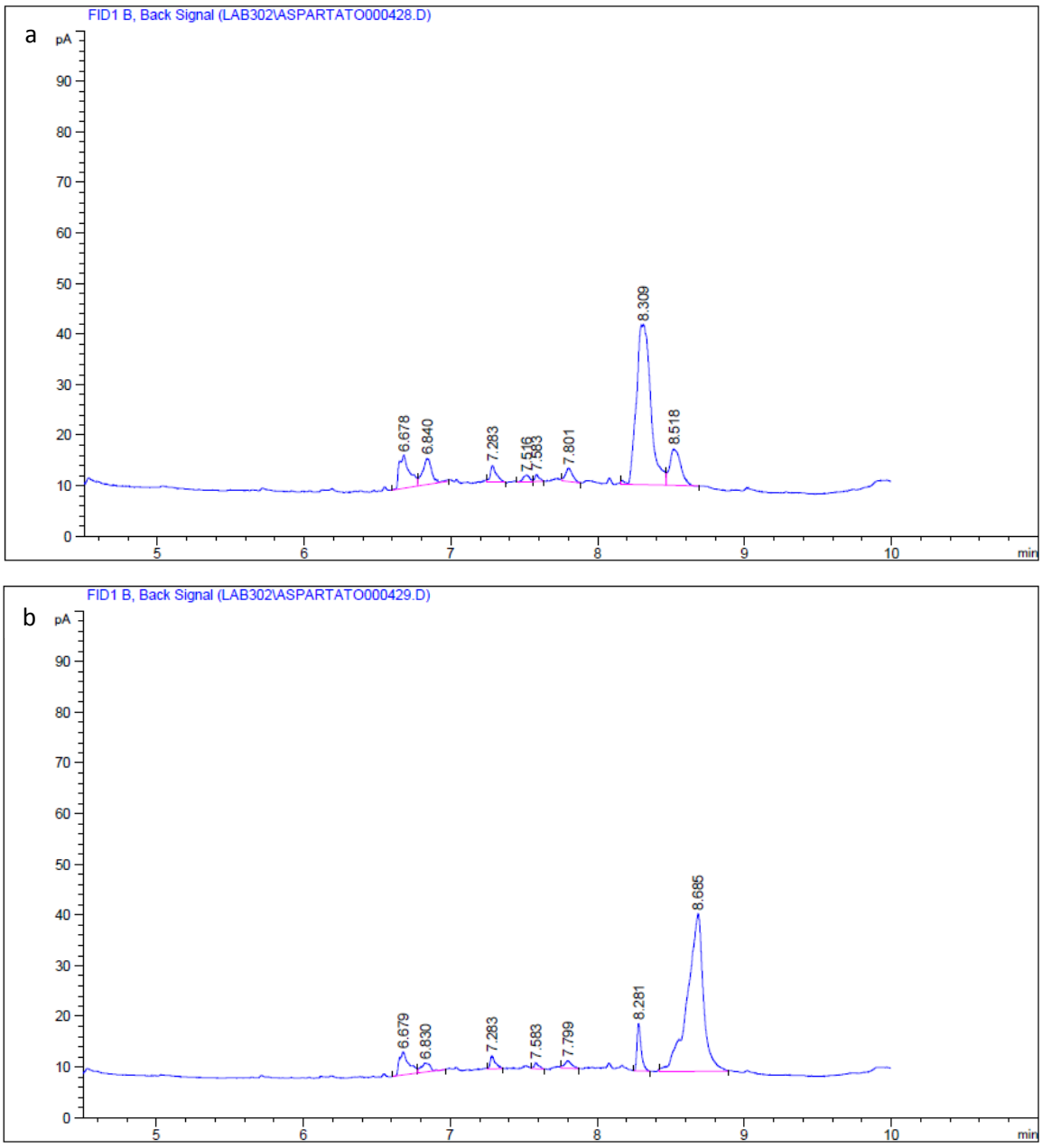


Figure 11: Chromatograms acquired in order to identify peaks corresponding to (a) L-Asp (8.309 min) and (b) D-Asp (8.281 min). Samples injected into a GC-FID device with a chiral column.

After heating different L-Asp standards at 112 ºC, 116 ºC, 120 ºC, being derivatized and injected as explained in previous sections, it could be observed that a downward trend was followed by peak areas. This supports the kinetic behaviour of any chemical being degraded at high temperatures. Nonetheless, the main pitfall observed was that a decrease of L-Asp area was not accompanied by an increase of D-Asp area in the same chromatogram, hence isomers were studied separately. After obtaining peak areas and heights for L-Asp, an exponential decay was observed according to results presented next, both graphs and equations together with correlation coefficients.

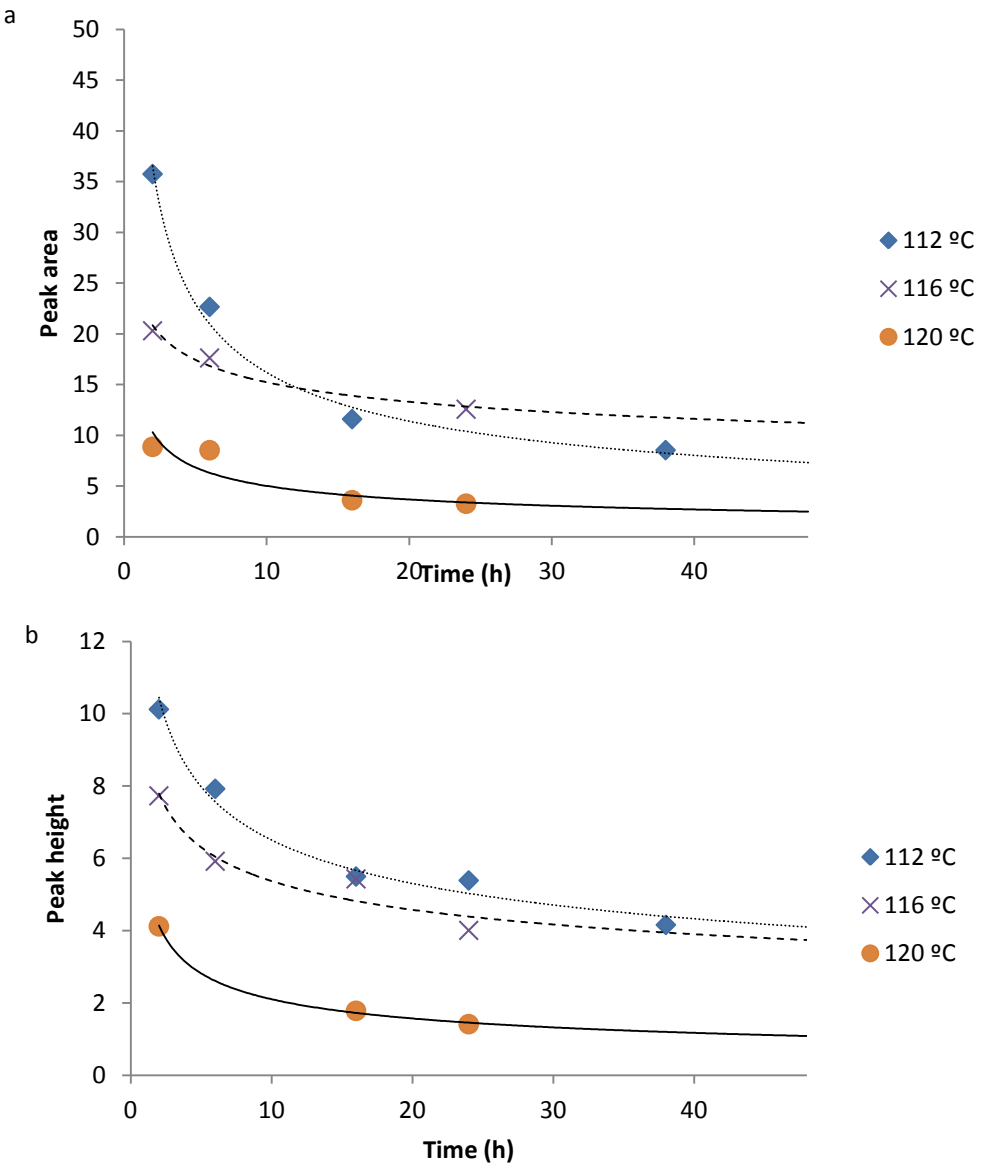


Figure 12: Decay trend of L-Asp as a high temperature is applied over time. Peak area (a) and peak height (b) were obtained after injecting several standards of this chemical into a GC-FID system with a chiral column.

Tabla 3: Exponential decay of L-Asp peak areas at different temperatures after injecting standards of this chemical into a GC-FID system with a chiral column. Equation followed is $y = Ae^{x}$.

Temperature (ºC)	A	x	R^2
112	51.887	-0.506	0.9865
116	23.863	-0.195	0.9729
120	14.074	-0.448	0.8509

Results are not very reliable as there was only one point for each temperature and time. It was not possible to analyze replicates of the standards due to a contamination of the chiral column, likely to have been with water coming from the aqueous phase of the tubes from which 1 µL of the organic phase (at the bottom) was injected. Therefore, a minimum of three replicates should be analyzed to improve the reliability of these results, which however show a clear degradation of L-Asp at a high temperature over time. Besides, starting point of this decay is more noticeable as long as temperature increases since it is easier to degrade more amount of L-Asp with higher temperature in a short time period.

The same procedure was applied to D-Asp at the same temperatures and times, results being shown below.

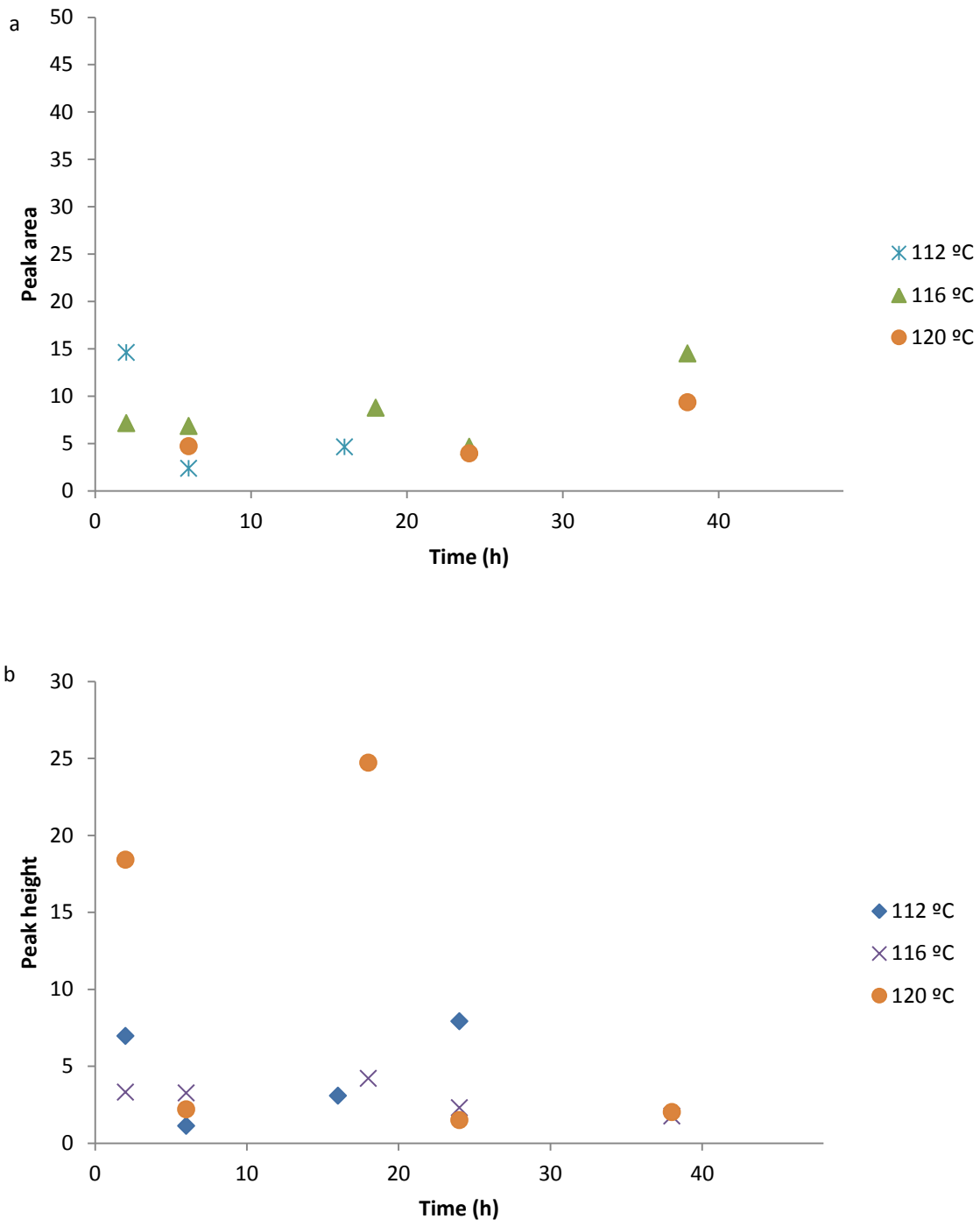


Figure 13: D-Asp evolution over time when a high temperature is applied. Peak area (a) and peak height (b) are directly proportional to D-Asp concentration and were obtained after injecting several standards of this chemical into a GC-FID system with a chiral column.

No trend was observed and it was not possible to predict the behaviour of this chemical with temperature. As explained in section 2.3, for this stage of the research a L-chiral column was utilized and the explanation of these unpredictable results might be related with a different sensitivity of a L-chiral column towards a D-isomer, because the latter seems to increase as time goes by when a high temperature is applied. If this happened, the reason for the good results with L-Asp would be explained because the column is very sensitive towards L- isomer and its decay could be monitored in a more reliable way. In the case of D-Asp a decay with temperature would be happening but the results showed an upward trend, probably related to an increase of L-Asp as D-Asp was decreasing and this isomer is more noticeable in the graph shown above. Another fact that needs to be considered is that these experiments, carried out at high temperatures, were intended to obtain chromatograms with two peaks corresponding to D- and L-isomer separately but actually this did not happen. A single peak at the same retention time was always appearing and this event might be explained with the low sensitivity that the GC-FID system achieves. With a more sensitive device (namely MS with any modality of mass analyzer) peaks would have been separated because a higher split could have been selected and this could have been promote narrower peaks, accompanied by an adequate separation of D- and L- aspartic acid isomers. Ratio between them could have been calculated and racemization assessment in fingermarks could have been achieved.

Thus L-Asp was the only isomer with which a kinetic study could be conducted and, bearing in mind that its racemization is a first-order reaction, the integrated equation will be: $[A] = [A]_0 e^{-kt}$, [A] and $[A]_0$ being concentrations at any time of the reaction and at the starting point, respectively, k being the rate constant of the reaction (k_{Asp}) and t being time. Taking natural logarithms, the equation is converted into: $\ln[A] = \ln[A]_0 - kt$, which follows a trend of a straight line ($y = ax + b$), where y is going to be ln[A] and x is going to be time (t), in hours. As concentration data were not available, areas played the role of concentrations and only a pseudo-rate constant k' could be calculated from the slope of the straight lines plotted next.

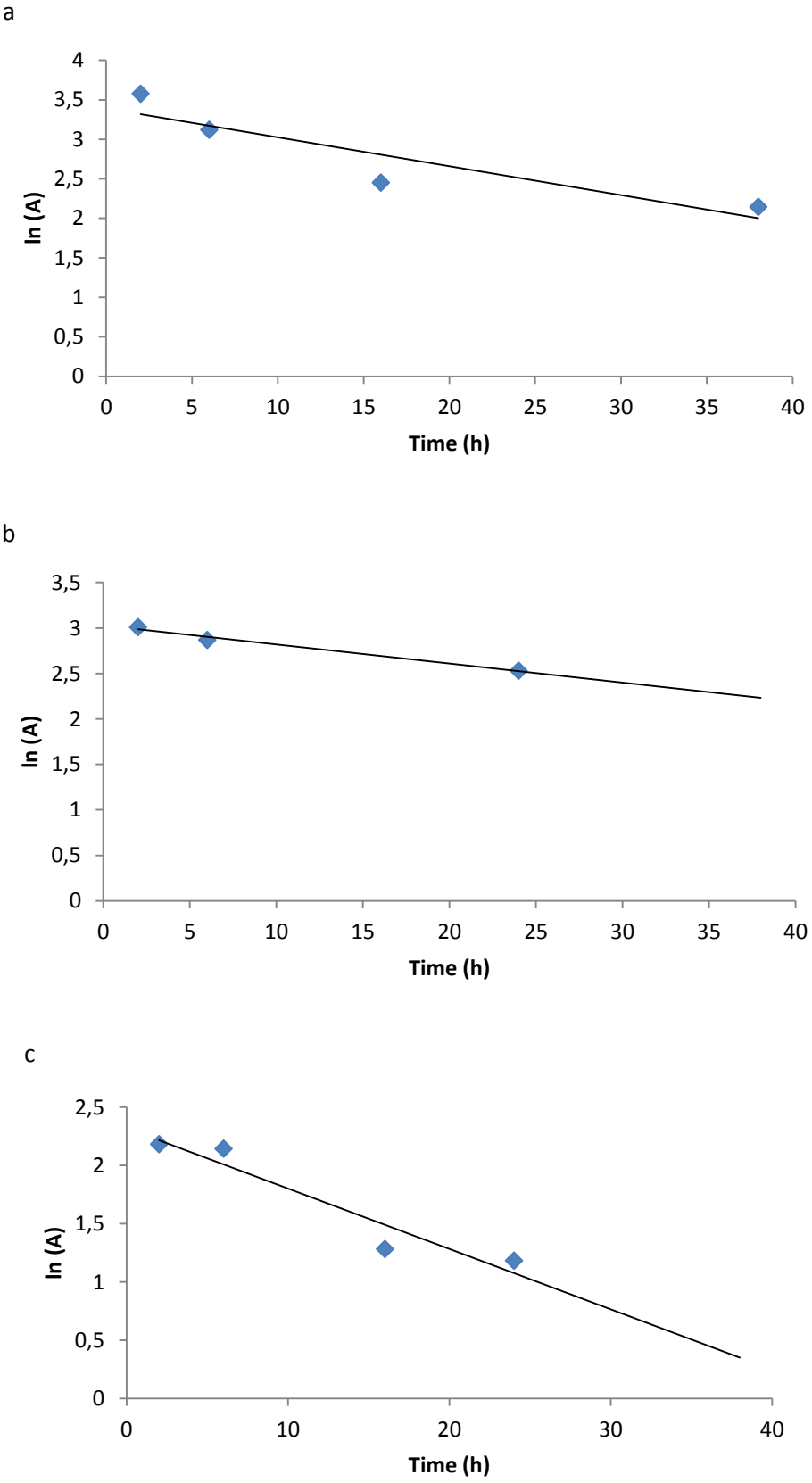


Figure 14: Kinetic study of L-Asp decomposition at (a) 112 ºC (b) 116 ºC and (c) 120 ºC when different L-Asp standards were injected into a GC-FID system with a chiral column. ln (A) refers to natural logarithm of peak areas for L-Asp.

Table 4: Determination of pseudo-rate constant for L-Asp after different peak areas were gathered for L-Asp standards injected into a GC-FID system with a chiral column.

Temperature (ºC)	k' (h^{-1})	R^2
112	0.0365	0.8278
116	0.0209	0.9857
120	0.0519	0.9152

As commented for peak area graphs, three replicates should be injected and the chiral column should show an adequate response for them all. Pseudo-rate constant seemed to increase with temperature, a requirement always met in kinetics of any reaction, but the first value should have been lower than the second. Correlation coefficient of the former explains its non-reliability and perhaps a better correlation could have provided with a lower figure which supported a likely upward trend of k'.

Besides, a calibration curve should be built for both L-Asp and D-Asp, analyses being conducted with the chiral column employed in this section. For this purpose, an internal standard should be used but it was not possible in this study due to the non-availability of a single isomer of (DL)-norleucine, the internal standard with which chromatograms in section 3.3 were performed. However, the initial thought was not using internal standard for this kinetic assessment as both isomers of aspartic acid would have been employed to calculate their ratio, which would have minimized reproducibility problems derived from manual injection. If (DL)-norleucine had been used with the chiral column, a double peak would have been obtained and it would have been more difficult to calculate ratio between internal standard and aspartic acid isomers.

3.4. AAR evaluation in fingermarks

Amongst fingerprints collected from the 19 individuals (referred in section 2.7) the first of them was injected and chromatogram acquired showed these results.

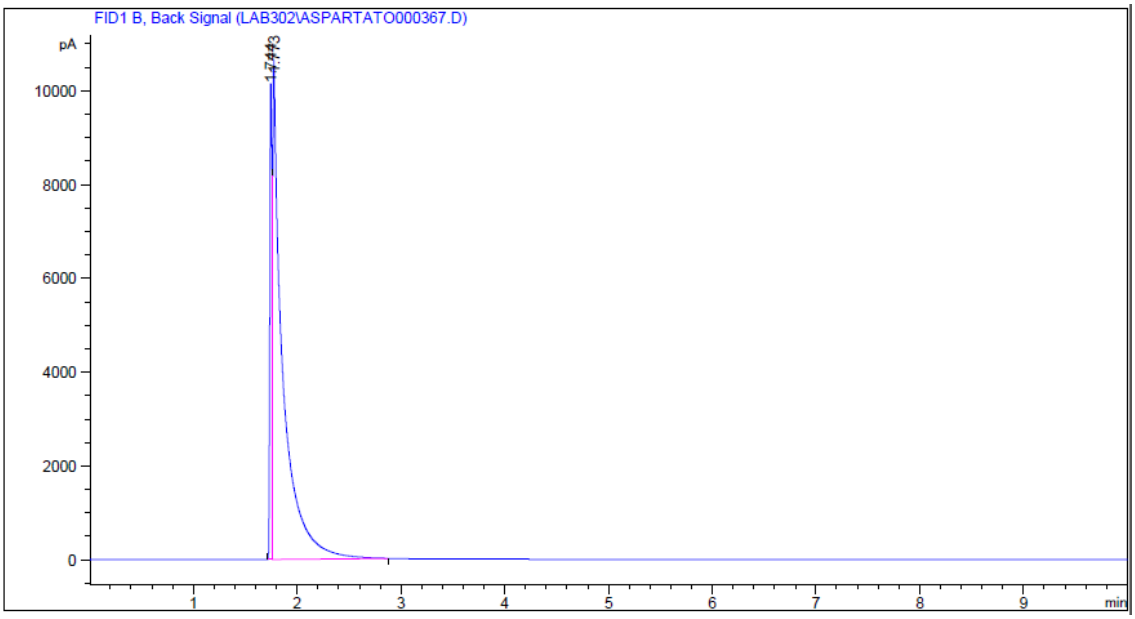


Figure 15: Fingerprint injected into a GC-FID device with a chiral column.

Chromatogram is shown in this view since no peak was found after the solvent even maximizing the scale. The only peak observed corresponded to the solvent (chloroform with 1% ECF). It is reasonable to believe that this failure in determining aspartic acid could have two reasons: 1) No sensitivity of the GC-FID device to quantify and even to detect the presence of aspartic acid; 2) Failure when applying the sample handling the following day and not the same day of collection, although all fingerprint samples were kept individually into small envelops or 3) Failure of the mere sample handling. The first conclusion could not be verified but the second and the third could.

As those fingerprint samples were not going to provide with any results, an alternative methodology was tested: a fingerprint was collected on one particular day and was treated on the same day of collection and an old fingerprint, taken one year before, was recovered from its storage. HCl 0.1 M was applied to 'wash' the small sheets onto which fingerprints were deposited and two beakers were utilized to collect the HCl enriched in chemicals from these fingermarks. For each of both samples, 120 µL were taken and placed into a Eppendorf tube, then heated at 116 ºC during 24 h. All the content evaporated but 60 µL of HCl 0.1 M reconstituted the sample and ECF-based methodology was applied. Chromatograms are shown below.

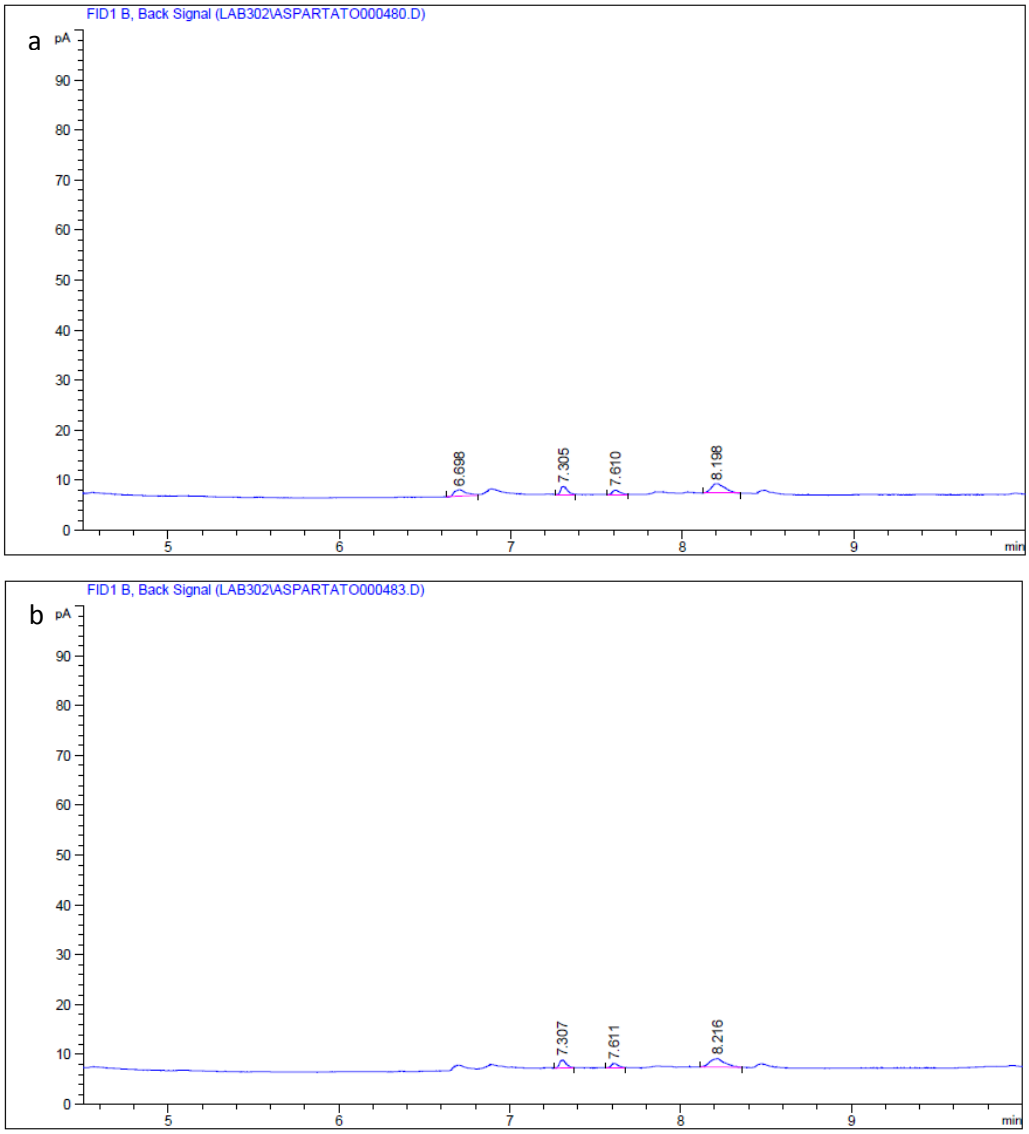


Figure 16: Chromatograms comparison between (a) a recent fingerprint, taken on the same day, and (b) an older fingerprint collected one year ago. Samples injected into a GC-FID device with a chiral column.

Chromatograms profile seemed to be the same and the time period passed since collection of the old fingerprint until the new one was collected and treated. Hence it may lead the researcher to two inferences: 1) Storage of fingerprints gathered initially should have been more rigorous or 2) Sample handling failed when attempting to enhance the effects of aspartic acid as the compound to be seeked in fingerprints. The first deduction can be easily rejected as sample storage was very similar to the one applied by other researcher one year before the current study. The second reason might be justified with the simpler methodology applied for the old and the new fingerprint, thus sample treatment employed initially involved such a complexity that it was not possible to detect any chemical coming from the fingerprints collected.

Another reasoning that supports the non-influence of time passed between collection and handling is found in chromatogram profiles for both old and new fingerprints. The only factor that affected fingerprints composition was temperature (116 ºC) and, with a more sensitive device, it would be possible to quantify D- and L- isomers of aspartic acid since this high temperature will have speeded up the racemization process.

3.5. Applications in forensic science

This methodology, although being at early stages, may be applied to every kind of biological samples, apart from fingerprints, which show aspartic acid in their composition. When sample is deposited onto a surface, racemization will begin and an appropriate handling of the specimen, followed by a subsequent analysis, as commented above, will provide with the information about time passed since deposition. In the case of fingerprints, this may be useful to predict when the fingermark was printed at the crime scene of many sorts of crimes (murders, rape, burglary, fraud...) in which hands were involved and no gloves are used to perpetrate the crime. With further research, this might become reliable evidence to present towards court in a trial session and it might be conclusive to blame or to reject a suspect.

4. Conclusions

An adequate calibration curve was obtained to quantify L- and D- isomers of aspartic acid but it was not possible to apply the methodology in fingerprint samples due to a failure of the chiral column when attempting to separate both isomers and the lack of a single-isomer internal standard.

AAR evaluation was not achieved in this study due to the non-adequate functioning of the chiral column employed, which did not provide with distinguishable peaks for both aspartic acid isomers. Instead, it was possible to conduct a kinetic study to predict the behaviour of L-Asp when temperature is increased and it was observed that a decay trend was maintained over time. A pseudo-rate constant was calculated but it was only based on peak area data and concentrations could have given a more accurate value of this kinetic constant. However, this rate constant gave an idea about the effect of high temperature on degradation of L-Asp, which did not happen with D-Asp since no trend was found according to results.

Because of the baseline fluctuations produced during the kinetic study, replicates were not injected and this is the reason behind the poor correlation coefficients obtained for the equations. Besides, no many points could be achieved and graphs are not very representative of trends found in the case of L-Asp.

First sample handling methodology failed when attempting to enhance the effect of aspartic acid in fingermarks collected and chromatograms showed no peaks. This was the reason to choose a simpler protocol, which was proven to work effectively to detect some chemicals in fingerprints, although aspartic acid was not detected given the low sensitivity of the GC-FID device. In addition, a recent and an old fingerprint were analyzed and time between collection and treatment did not seem to influence chemical composition as chromatograms profile happened to be quite similar.

5. Recommendations for further work

This is a preliminary methodology and further research is a necessity, a sensitive technique being the primary aspect to take into account. Since it was discovered and demonstrated, GC-MS has always been the desirable technique to analyze amino acids in fingerprints due to the high sensitivity it shows to detect and quantify them at the extremely low levels expected to be found in this kind of samples (in the region of hundreds of ng). Hence all fingerprint analyses should be performed on a GC-MS device thanks to its confirmatory power, which will provide with the identity of the compound, and to obtain a noticeable peak for aspartic acid.

Fingerprint collection shall consider more individuals to obtain more accurate and reliable results within each of the variables taken into account in this research (age, ethnicity, diet...). In this way a Principal Component Analysis (PCA) could be performed to evaluate differences in fingermarks composition across all the ethnic groups studied.

Aspartic acid is not the most abundant amongst amino acids present in fingerprints, therefore it would be advisable to conduct a fingermark aging study with special attention to the most predominant amino acids: serine, glycine and alanine, aspartic acid being the fourth in abundance. Their racemization will not be as fast as in the case of aspartic acid but they could be recovered to a greater extent, providing a suitable derivatization method is applied, due to their abundance.

A good functioning of the chiral column should be ensured to separate both stereoisomers of aspartic acid and, in this way, it will be possible to determine ratio between them. If both peaks could not be resolved, a single-isomer internal standard should be employed to minimize manual-injection imprecisions. It is recommendable to build a calibration curve with the chiral column, as done with the non-chiral one, to quantify the amount of D-Asp and L-Asp in fingermarks and it would be likely to go further in this approach and to obtain the molecular clock stated in the title of this project.

6. References

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7. Appendices

7.1. GC-FID device

Figure 17: GC-FID device with front view (top left and right), inside view (bottom left) and injector (bottom right).

7.2. Columns utilized for analyses

Figure 18: Chirasil L-Val column (left side) and HP-5 column (right side) installed in the GC-FID device seen above.

7.3. Samples prepared and injection syringe

Figure 19: Eppendorf tube with a sample derivatized (left side) in which aqueous phase is on top and organic phase (chloroform with reagents and analyte) at the bottom; syringe for injection (right side).