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NUCLEAR MAGNETIC RESONANCE AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CHEMICAL ANALYSIS OF PERUVIAN ROASTED COFFEE BEANS

Tesis para optar el Título de **MAGÍSTER EN QUÍMICA**, que presenta:

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ABSTRACT

Coffee is the most traded agricultural commodity in the world. Currently, Peru is considered the third principal producer of *Coffea arabica* in South America, and the sixth worldwide, accounting for 6 % of the global production. However, most of the coffee (~ 99 %) is exported as green beans due, in part, to the fact that the local quality control of the roasting process is not yet optimal. Hence, it is crucial to develop a more standardized process for the quality control of roasted coffee beans that will allow local Peruvian coffee farmers to introduce to the market a more valuable product.

In the thesis presented here, work associated with the project FINCyT-PIPEI-PUCP-CENFROCAFE-2012 on the quantitation of the main compounds developed during the roasting process was embraced using NMR and HPLC-DAD methodologies. Attention was focused on the secondary metabolites that, from a flavor-aroma perspective, are of interest: caffeine, 5-caffeoylquinic acid**,** trigonelline, 1-methylpyridinium ion and nicotinic acid, and 5-hydroxymethylfurfural as a marker of deterioration.

One- and two-dimensional NMR techniques allowed the simultaneous identification of eleven compounds known to be associated with the flavor and aroma of coffee. The NMR quantitation of five compounds was performed using ERETIC2 and Standard Calibration Curves and the results were validated by a new HPLC methodology, which constitutes the only validated methodology currently available for the simultaneous quantitation of these five compounds. The percentage difference among this three methods was within acceptable values (1 – 20%) for most of the compounds. It was demonstrated that these numbers were sample dependent. In addition, PCA analyses of quantitative data (NMR and HPLC-DAD) allowed the discrimination of coffee samples acording to the degree of roasting, as well as to their origin (instant coffee, speciality coffees from different regions in Peru).

Hence, these preliminary results indicate that NMR and HPLC can be used as quality control tools to optimize the roasting conditions of Peruvian specialty coffee. Recommendations are included in this work to improve further the error percentages between the NMR and HPLC data that in some cases (for 5-caffeoylquinic acid and nicotinic acid) were high.

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I hope this will contribute with the work of the CENFROCAFE team and also to encourage them to keep practicing organic agriculture as a sustainable alternative to the extractive industries which are jeopardizing the delicate equilibrium of fragile environments like wetlands and basin heads in Cajamarca.

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LIST OF ABBREVIATIONS

^{13}C-NMR	Carbon Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
5-CQA	5-Caffeoylquinic acid
5-HMF	5-Hydroxymethylfurfural
C. arabica	*Coffea arabica*
C. canephora	*Coffea canephora*
CGA	Chlorogenic acids
COSY	Correlation Spectroscopy
CQA	Caffeoylquinic acids
D_2O	Deuterated water
ERETIC2	Electronic Reference to Assess in Vivo Concentration
ESI	Electrospray Ionization
Et_3N	Triethylamine
FAO	Food and Agricultural Organization of the United Nations
GC-MS	Gas Chromatography – Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation Spectroscopy
ICO	International Coffee Organization
INIA	National Institute of Agrarian Innovation (Government of Peru)
LC-MS	Liquid Chromatography – Mass Spectroscopy
LB	Line Broadening
MeOH	Methanol
MINAG	Ministry of Agriculture (Government of Peru)
NMR	Nuclear Magnetic Resonance
NOEs	Nuclear Overhauser Enhancements
NOESY-Presat	Nuclear Overhauser Effect-Presaturation
NS	Number of Scans
PCA	Principal Component Analysis
PLS	Partial Least Squares fitting
PULCON	Pulse Length based Concentration Determination
qNMR	Quantitative Nuclear Magnetic Resonance
SCAA	Specialty Coffee Association of America
SCAE	Specialty Coffee Association of Europe
SIICEX	Integrated System of Trading Information (Peru)

T_1	Longitudinal Relaxation Time
T_2	Transverse Relaxation Time
TOCSY	Total Correlation Spectroscopy
TSP	3-Trimethylsilyltetradeuteropropionic acid sodium salt

1. INTRODUCTION: BACKGROUND AND SIGNIFICANCE OF THE STUDY

1.1. Characterization of coffee

Coffee (*Coffea* spp.) is a tropical plant indigenous to Africa that belongs to the Rubiaceae family. Coffee trees are characterized by a great morphological variation, which complicates its taxonomic classification (Clifford and Willson, 1985). Today, coffee is widely grown throughout the tropics, principally in Latin America (FAO, 2011, ITC, 2011).

The plant is characterized by a pronounced dimorphism between upward and horizontal growing branches. They tolerate shade and are amenable for agroforestry ecosystems because they share similar growth requirements with forest trees (Verheye, 2010). Coffee presents markedly stages of vegetative and reproductive growth with times between flowering and picking stages, which range from 6 to 11 months, depending on the coffee species (Verheye, 2010). The ripe fruit has a red or purple color, and usually contains two seeds (green coffee beans). Its production requires specific edapho-climatic conditions with altitude, temperature, rainfall, sunlight, and soil being the most influential factors (Verheye, 2010). Rich soils, moist climate, high altitudes and middle temperatures contribute to the flavor of the bean, leading to the highest quality coffee (Cansonni *et al*., 2012).

The international coffee trade is principally centered in two coffee species, *Coffea arabica* and *Coffea canephora* (synonym *C. robusta*) (Rubiaceae), which account for around 64 % and 35 % of the global production, respectively (Verheye, 2010). It is widely accepted that *C. arabica* produces a superior taste and consumer acceptance than *C. robusta*, which is considered a lower quality species (Jaiswal *et al*., 2010; Cansonni *et al*., 2012). High quality coffee blends consist of 100 % of *C. arabica* imposing higher market prices (Jaiswal *et al*., 2010). However, the cultivation of *C. robusta* has some advantages over that of *C. arabica* including, faster growing and adaptation to a larger variety of climatic conditions and altitudes (Jaiswal *et al*., 2010), and higher resistance to leaf rust, a devasting desease for coffee plantations caused by the fungus *Hemileia vastatrix* (Verheye, 2010).

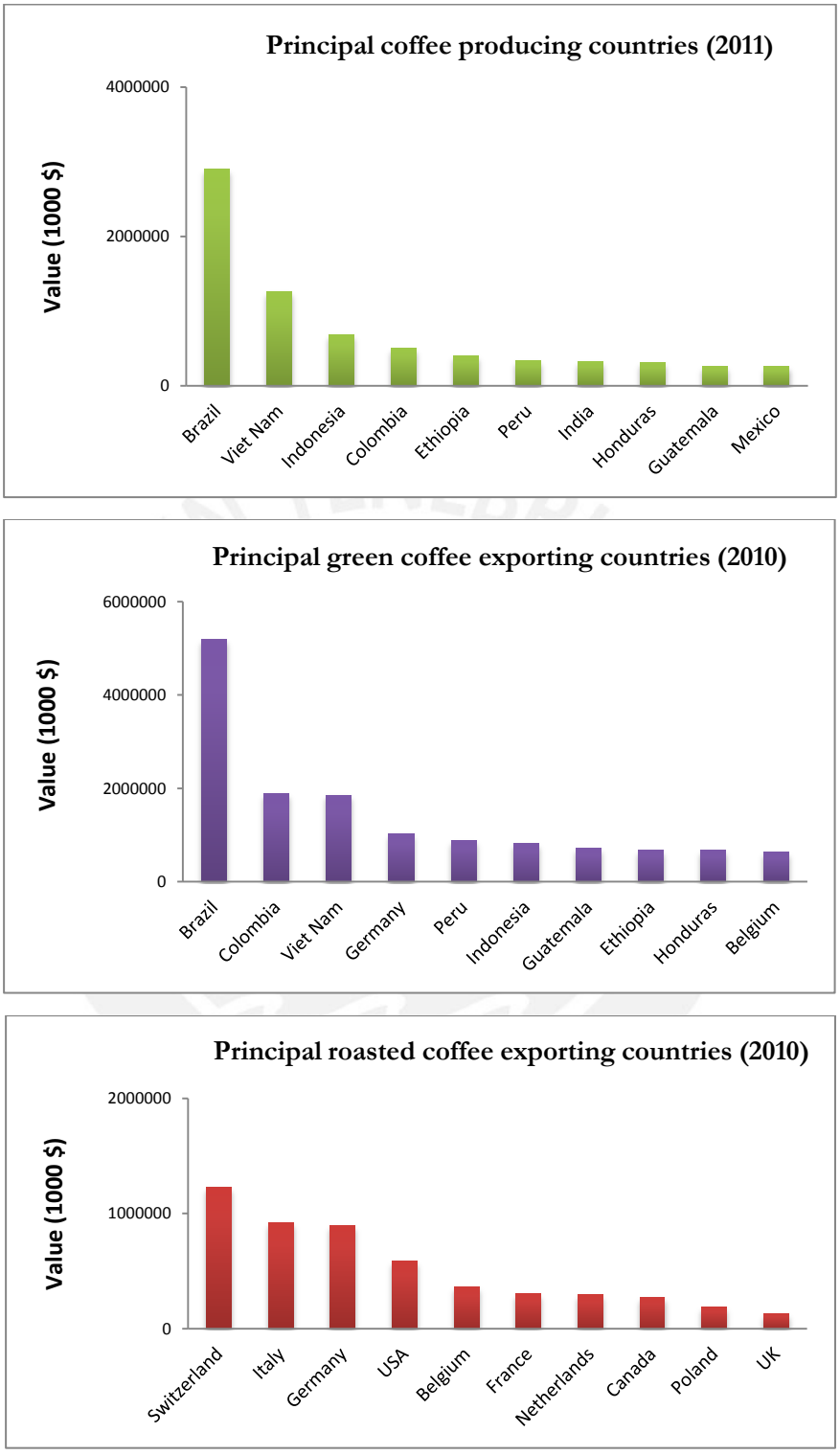


Figure 1.1. World coffee trade and production (source: FAO, 2011).

1.2. Global situation of coffee

Coffee is the world´s most traded agricultural commodity, being a vital contributor to the economy of several countries[1] (ITC, 2011). It has been estimated that about 26 million people, from 52 countries, depend on coffee production for their livelihood (Castro, 2004).

The principal coffee producing and exporting countries are shown in **Figure 1.1** (FAO, 2011). As can be seen, the bulk of the world coffee production is concentrated in Latin America, with Brazil being by far the largest grower and seller in the world. Between the principal importing countries, the United States represents the first market for green coffee (data not shown, FAO, 2011). Interestingly, none of the principal roasted coffee exporting countries are coffee producers.

1.3. Coffee in Peru: Specialty coffees

Coffee has become a major source of income for Peru, and accounts for over 30 % of the agricultural export basket (MINAG, 2012). Moreover, it plays a significant social role, as it is involved in a national strategy to defeat drug trafficking in the Amazon region, by the replacement of coca cultivars employed for the production of cocaine (INIA, 2012; MINAG, 2012).

Peruvian coffee is well-appreciated worldwide for its unique flavor and aroma due to the privileged environmental conditions available throughout different regions of Peru, one of the seventeen megadiverse countries in the world. Coffee is cultivated in sixteen regions within the country. However, the most suitable environment, in terms of geographical and climatic conditions favorable for the cultivation of coffee, is the area east of the Andes, characterized by the presence of dense and rainy mountain forests. Given this feature, coffee production is basically concentrated in five regions: Junin, Cajamarca, San Martin, Amazonas and Cuzco, in order of importance, which account for 92 % of the national production (**Figure 1.2**). As a result of this privileged environment, Peru is well-known for its suitability for the production of specialty coffees, which constitute a particular class of coffee characterized by a unique quality, different and superior from the common coffee beverages. This denomination is associated with the high standards involved in the production and handling of the green bean (SCAE). Yet, little

[1] In Uganda and Burundi, for instance, 90 % of their gross national product (GNP) is based on coffee exports (Wintgens, 2009).

is known about the chemical and physical properties of Peruvian coffee that are responsible for its unique quality.

Currently, Peru is considered the third principal producer of coffee (*C. arabica* is the only cultivated coffee species) in South America, and the sixth worldwide (**Figure 1.1**), accounting for 6 % of the global production (MINAG, 2011). However, most of the coffee (~ 99 %) is exported as green beans (SIICEX, 2011) due, in part, to the fact that the local quality control of the roasting process is not yet standardized (ESAN, 2010). This fact represents a substantial reduction of profit margins for national farmers considering that the price of the roasted product is significantly higher than that of the green bean.

Peru: Principal coffee producing regions

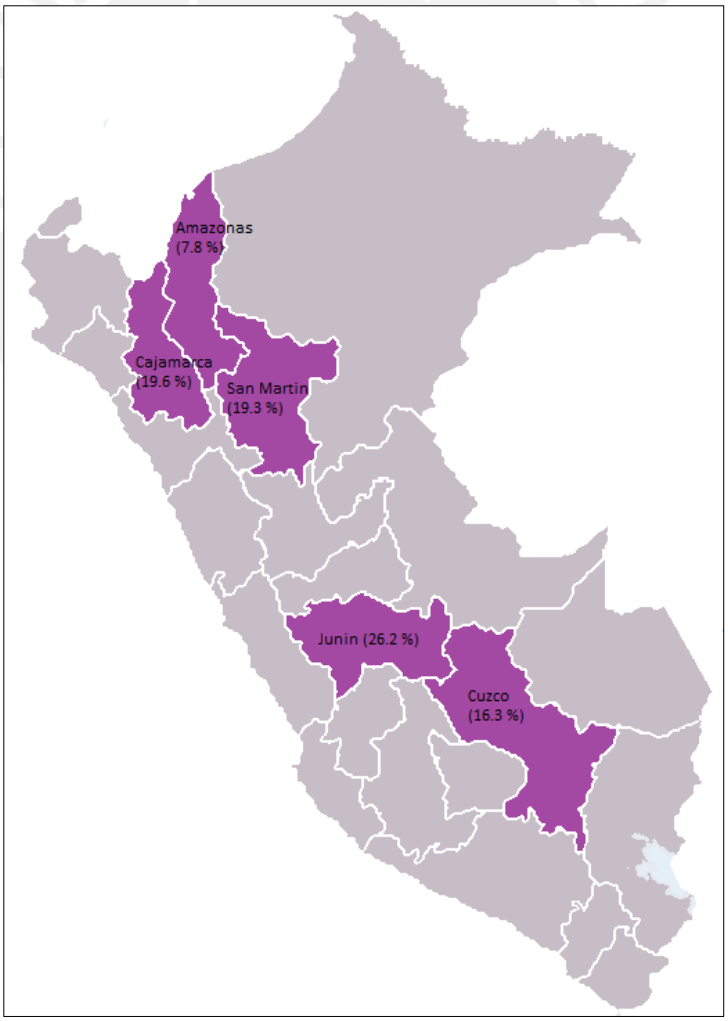


Figure 1.2. Coffee producing regions in Peru (source: MINAG, 2011).

Given the social and economic importance of coffee in Peru, it is crucial to develop a more standardized process for the quality control of roasted coffee beans that will allow local Peruvian coffee farmers to improve their roasting protocols. In the next section, the physical and chemical changes of coffee known to take place during the roasting process will be described, with special emphasis in the evolution of particular compounds that have been reported as quality markers.

It is important to mention that, as opposed to Brazil and Colombia, the two main Latin American coffee producers, in Peru, no center, institution, or laboratory participates in the chemical quality control of coffee, green or roasted. The first example of a combined effort between academia and a local coffee producer is the collaboration established by the Pontificia Universidad Catolica del Peru and CENFROCAFE (Maruenda *et al.*, 2012). Both entities are interested in establishing a quality control protocol beyond the one centered in good agricultural practices and in basic physical properties of green beans, such as humidity and integrity of the grain.

In this collaborative effort, the main goal is to optimize the roasting protocol available in CENFROCAFE. Hence, in the thesis presented here, work that belongs to the first stage of the project, the quantitation of the main compounds developed after roasting, and associated with flavor and aroma of a cup of coffee, will be embraced.

1.4. Influence of the roasting process on coffee chemical composition

The roasting process is key in the development of the characteristic flavor and aroma of coffee, features which are absent in the green coffee (Clarke and Vitzthum, 2001; Wei *et al*., 2012 a). The quality of the green bean and its inherent chemical composition, both associated with environmental and harvesting conditions, are also determinant factors in the quality of the final product.

During roasting, many complex chemical and physical changes take place, all of which depend on the particular conditions of the process (temperature, time, load and weight of the batch). Drastic roasting conditions (excessive time and heating temperatures) are known to cause undesirable chemical changes in the bean (Casal *et al*., 2000a) leading to a strong and unpleasant bitter taste (Blumberg *et al*., 2010).

The main aspects of the roasting process are illustrated in **Figure 1.3**. Inside the roaster, exothermic and endothermic reactions take place, leading to gas formation, mainly H_2O and CO_2,

and to an increase of the internal bean pressure to finally cause the cellulose cell wall of the bean to crack (Clarke and Vitzthum, 2001). Heat energy transportation into the coffee bean, and therefore the technology applied in this process (drum or fluidized bed reactors), determines the rate and uniformity of roasting (Clarke and Vitzthum, 2001). As temperature increases, Maillard reactions (sugar degradation) give rise to the formation of melanoidins, colored polymers responsible for the gradual darkening of the bean (Clarke and Vitzthum, 2001).

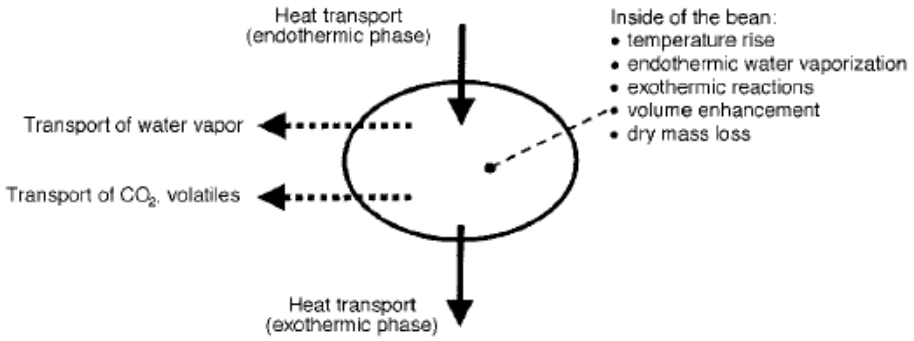
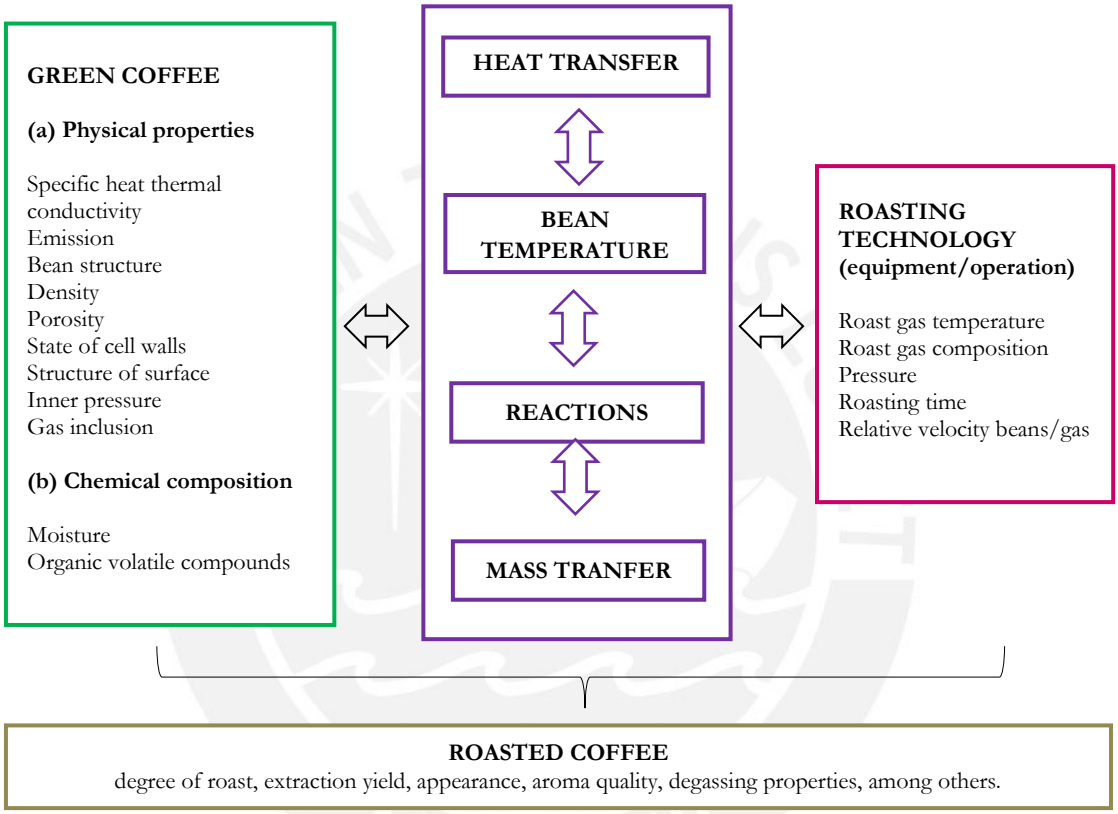


Figure 1.3. Main aspects of the roasted coffee bean process (reproduced from Clarke and Vitzthum, 2001).

Scheme 1.1 illustrates the principal processes taking place during roasting associated with the properties of the starting material (green bean) and the end product (roasted bean). The time and temperature at which the green beans are heated have a crucial influence on the particular characteristics of the final product, since they will have a strong effect on the chemical reactions taking place through roasting, as also do the physical properties and chemical composition of the starting material. This high interdependence makes the process complex to study, and hence the physical and chemical mechanisms taking place during roasting are not yet fully understood (Clarke and Vitzthum, 2001**)**.

As mentioned earlier, the roasting process gives rise to complex chemical changes in the green bean composition. Several chemical reactions occur simultaneously leading to the degradation of polysaccharides, chlorogenic acids and amino acids, among many other metabolites, and to the formation of lipids, low molecular weight organic acids, as well as many others (Ciampa, 2010; Wang, 2012). There are some compounds present in the green bean - quinic acid for example- which increase in concentration during roasting. In the case of quinic acid this is due to the degradation of chlorogenic acids.

Various reports studying the changes in chemical composition of coffee (*C. robusta* and *C. arabica*) during roasting have recently become available in the literature (Yen *et al.*, 2005; Trindade *et al.*, 2006; Moon *et al*., 2009; Blumberg *et al*., 2010). They are concerned mainly with the polysaccharides and caffeoylquinic acids in the case of the green beans, and the chlorogenic acids, trigonelline, caffeine and nicotinic acid in the case of the roasted beans.



Scheme 1.1 Coffee roasting process, its interrelation with crude coffee, roasting technology and its effect on the end product (adapted from Clarke and Vitzthum, 2001).

In this study, attention will be focused on the secondary metabolites that, from a flavor-aroma perspective, are of interest at the initial stage of the project PUCP-CENFROCAFE, some of which are associated directly with the degree of roasting: caffeine, caffeoylquinic acids**,** 1-methylpyridinium ion and nicotinic acid. Trigonelline is also assessed, and as a marker of deterioration (del Campo *et al*. 2010), 5-hydroxymethylfurfural content is analyzed. The techniques used for quantitation in this study are not the most convenient to address quinic acid. In the case of caffeic acid, previous studies (Maruenda, 2009) have shown that this molecule is absent in specialty coffees.

1.4.1. Main alkaloids present in coffee: Caffeine and Trigonelline

Caffeine is a water-soluble, purine alkaloid which is, by far, the most studied compound in coffee, due to its physiological effects. It is also found in other important beverages as cocoa, maté, guarana and tea (Dewick, 2009). The structure of caffeine (**1**) is shown in **Figure 1.4**. It is a tri-*N*-methyl derivative of xanthine that acts as a competitive inhibitor of phosphodiesterase (Dewick, 2009). This enzyme participates in the degradation of cyclic AMP (cAMP) leading to the stimulation of the central nervous system by mimicking the action of catecholamines (Dewick, 2009). Hence, due to this effect, the recommended maximum daily intake should not exceed about 1 g to avoid disagreeable side effects and an intake of 5-10 g is considered lethal (Dewick, 2009).

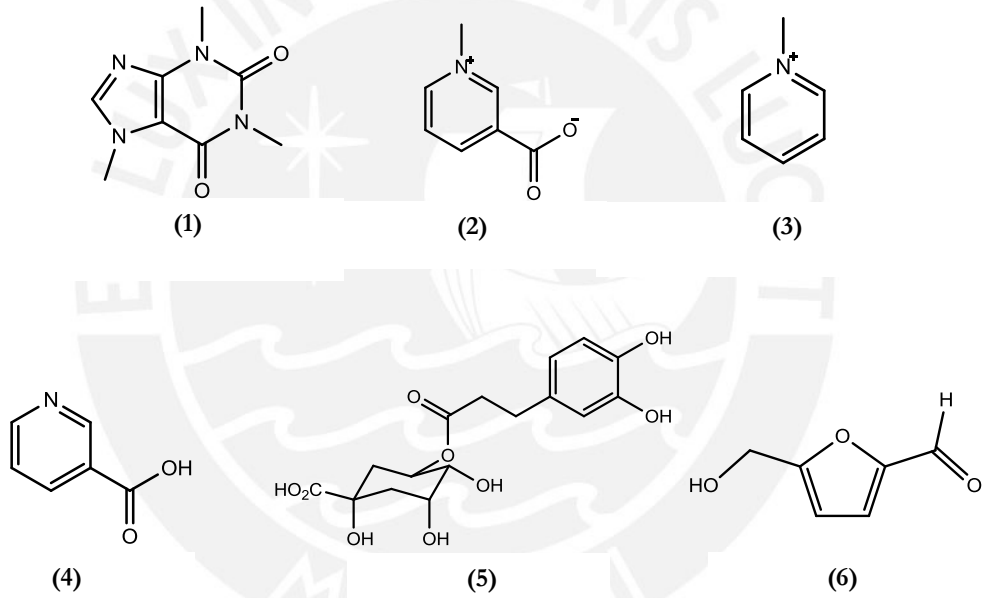


Figure 1.4. Chemical structures of caffeine (1), trigonelline (2), 1-methylpyridinium ion (3), nicotinic acid (4), 5-caffeoylquinic acid (5) and 5-hydroxymethylfurfural (6).

Green coffee beans contain between 1 to 2 % of caffeine. The amount depends on the coffee species: *C. canephora* (syn. *C. robusta*) commonly contains more caffeine than *C. arabica* (Clarke and Vitzthum, 2001).

It is a consensus in the literature that the concentration of caffeine is only slightly affected during roasting. Most studies have reported a small decrease with longer roasting times and temperatures (Clarke and Vitzthum, 2001). However, Moon *et al*. have recently reported a slight increase of caffeine with more severe roasting conditions in all seven brands they studied (Moon *et al*.,

2009). Caffeine is found in the green bean mainly combined with chlorogenic acids through hydrophobic π complexes (D´Amelio *et al*., 2009). This could explain its relative stability upon heating.

Trigonelline (**2** in **Figure 1.4**) is a pyridine alkaloid, biosynthesized from nicotinate, and is known to be widely distributed in plants (Stadler *et al.*, 2002, a). Its importance is related to the known anticarcinogenic (cervix and liver), hypocholesterolemic and glucose-lowering activities, among other therapeutic properties (Zeiger, 1997, Lang, 2010). Trigonelline has been claimed as the second most abundant alkaloid in green coffee after caffeine (Stadler *et al*., 2002 b). However, unlike the latter, it is present in *C. arabica* (7.9 - 10.6 g / kg) in higher amounts than in *C. canephora* (robusta) (6.6 - 6.8 g / kg) (Stadler *et al*., 2002 b). Also, unlike caffeine, it undergoes significant degradation during roasting; forming a wide variety of aromatic nitrogen compounds related to brew bitterness and, in consequence, is considered a key precursor of coffee flavor and aroma (Casal *et al*., 2000 a; Stadler *et al*., 2002 a,b). It has been also suggested that trigonelline is, by itself, an important contributor to bitterness through combination with cyclic peptides and aminohexose reductones (Clarke and Vitzthum, 2001).

Moreover, it has been shown, when comparing trigonelline hydrate, trigonelline hydrosulfate and trigonelline hydrochloride degradation, that the nature of the counter ion in trigonelline reactions is a determinant factor in defining the reaction pathway for its degradation (see **Figure 1.5**), and in consequence it should be taken into account in the evaluation of trigonelline (Stadler *et al*., 2002 b). This will undoubtedly also have an impact on the flavor-aroma profile of coffee given that alkylpyridinium products may indeed be significant contributors to the bitterness of the coffee brew (Stadler *et al.*, 2002 b).

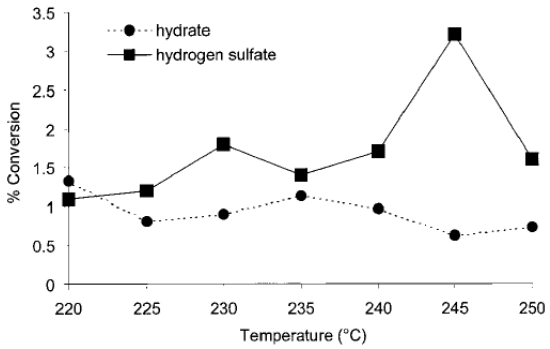


Figure 1.5. Conversion of trigonelline salts (hydrate, hydrogen sulfate) to give alkylpyridinium products as a function of temperature, at a constant pyrolysis time (15 min). Values are expressed as percent of conversion of trigonelline on a molar basis (Stadler *et al.*, 2002 a).

Casal *et al.* (2000 a) performed an investigation of the thermal degradation of trigonelline and caffeine in *C. arabica* and *C. canephora*. In this study, the trends and patterns previously mentioned for these compounds, in terms of variation among species and thermal behavior, can be appreciated (results are shown in **Table 1.1**). A slight decrease of caffeine concentration and a decrease of trigonelline content, which become significant when the temperature reaches 240 °C, were observed.

Table 1.1 Results (mg/kg dry mass base) for the roasting program performed at different temperatures for a constant time (15 min) (Casal *et al.*, 2000 a).

	Trigonelline		Caffeine	
	C. arabica	*C. robusta*	*C. arabica*	*C. robusta*
Green	8.91	6.32	12.36	20.84
140°C	8.47	6.37	14.37	22.12
160°C	8.31	5.86	15.18	21.71
180°C	8.29	5.78	13.57	19.81
200°C	7.80	5.43	13.87	19.93
220°C	5.57	4.20	12.95	19.88
240°C	0.49	0.97	10.96	19.25

1.4.2. Trigonelline decomposition products: Nicotinic Acid and 1-Methylpyridinium

Two major decomposition pathways operate for the thermal degradation of trigonelline: decarboxylation to afford the 1-methylpyridinium ion (**3** in **Figure 1.4**), as the major reaction product, and *N*-demethylation to give nicotinic acid (**4** in **Figure 1.4**) (Stadler *et al.*, 2002 a). In the study performed by Stadler *et al.*, the degradation pathway was demonstrated indirectly, by heating trigonelline at temperatures mimicking roasting, from 220 to 250 °C. The chemical structures of these compounds are shown in **Figure 1.4**.

1-Methylpyridinium ion is a non-volatile, alkylpyridinium compound poorly studied in natural sources due to its quaternary nature, a feature that complicates the isolation and quantitation protocols (Stadler *et al*., 2002 b). Even though 1-methylpyridinium cation is known to exist in various marine gastropods / bivalves, coffee is recognized as a major food source for this compound (Stadler *et al*., 2002 b), which is currently under investigation due to its potential anti-carcinogenic properties (Boettler *et al.*, 2011, Volz *et al*., 2012).

1-Methylpyridinium ion has been reported by Stadler *et al.* (2002 a), as the major product of trigonelline degradation during coffee roasting, reaching levels from 0.06 to 0.25 % due to the high polarity of the molecule, which may lead to human exposures of 30 to 125 mg / person / day. Like trigonelline and caffeine, it has been related to the bitter taste of coffee (Stadler *et al*., 2002 b). More recently, Lang *et al.* (2010) found high plasma levels of 1-methylpyridinium after coffee consumption, which demonstrates that this molecule is rapidly absorbed despite its cationic natures and that it effectively enters into the vascular system, an indispensable step for the biological *in vivo* effects claimed for this compound.

As opposed to previous studies, which reported immediate methyl rearrangements to yield pyridines and alkylpyridines, Stadler *et al*. (2002 a) found that 1-methylpyridinium is rather stable during roasting. According to these studies, the stability of 1-methylpyridinium depends on the nature of the standard trigonelline counter ion employed, suggesting that this might stabilize the quaternary amine. In the real coffee matrix, 1-methylpyridinium must be associated with organic or inorganic counter ions which fulfills this role, such as the phosphate, chloride, formate, lactate, or even with more complex molecules, such as the melanoidins (Stadler *et al*., 2002 b).

According to the studies performed by Stadler *et al.* (see **Table 1.2**), the formation of 1-methylpyridinium from trigonelline is strongly dependent on time and temperature, and reaches a maximum at 240 °C, after 20 min of roasting. This result is consistent with the data obtained by Casal *et al*. (2000 a), who reported a significant decrease in trigonelline content when the temperature reaches 240°C (see **Table 1.1**). This behavior suggests that the ratio trigonelline / 1-methylpyridinium could be used as an indicator of organic roasting loss (Stadler *et al*., 2002 b), and moreover, it may be useful to monitor the coffee roasting process, which is the aim of the present study.

The decrease in 1-methylpyridinium formation after prolonged roasting periods (>20 min) (**Table 1.2**) may be the result of further decomposition, rearrangement mechanisms or alkylation reactions with potential nucleophiles present in coffee (Stadler *et al*., 2002 a). The dramatic changes observed at 240 °C as compared to 220 and 230 °C, where after a significant decrease from 18 to 4 % at 20 min, an increase with longer roasting periods was registered, were not explained or even mentioned in the article.

Table 1.2. Formation of 1-methylpyridinium from trigonelline hydrochloride as a function of temperature and time. Expressed as percent conversion on a molar basis (adapted from Stadler *et al.*, 2002 a).

time (min)	220 °C	230 °C	240 °C
5	0.64	1.14	2.53
10	1.33	1.77	6.63
20	1.63	3.08	18.79
30	1.77	3.18	4.56
45	1.29	2.18	7.12
60	1.08	1.77	9.15

Nicotinic acid (**4** in **Figure 1.4**), also known as niacin or vitamin B_3, is an essential human nutrient biosynthesized through the oxidative degradation of tryptophan (Dewick, 2009). Its deficiency has been related to pellagra, a chronic disease associated with erythematous dermatitis and dementia (FAO, 2002). The consumption of coffee contributes to the minimum dietary requirements of nicotinic acid for an adult (Stadler *et al.*, 2002 a), which ranges from 14 to 16 mg / day (FAO, 2002). Although there is an endogenous amount of nicotinic acid in green coffee beans (8 – 17 mg / kg), its concentration is significantly augmented during the roasting process, mainly as a product of trigonelline demethylation (Casal *et al*., 2000 a; Stadler *et al*., 2002 a).

As in the case of 1-methylpyridinium, the formation of nicotinic acid has been correlated to the roasting degree through model studies that mimicked roasting (Casal *et al*., 2000 a). **Figure 1.6** indicates that *N*-demethylation (formation of nicotinic acid) is favored over *N*-decarboxylation to give 1-methylpyridinium, at temperatures in the range of 220-240 °C. Stadler *et al*. (2002 a) explain that this behavior may be the result of the quaternary nitrogen that favors methyl transfer reactions.

To evaluate the influence of heating time on the formation of nicotinic acid, Stadler *et al.* (2002 a) established six different time-periods for trigonelline hydrochloride thermal treatment. **Table 1.3** shows the results of this evaluation. A salient increase of nicotinic acid with time and temperature (an almost fivefold increase after only 5 min of reaction was observed at 220 °C) and good stability, even at high temperatures and long roasting times, was observed. In contrast to the pattern reported at 220 and 230 °C for 1-methylpyridinium (**Table 1.2**), nicotinic acid follows a constant increase over the entire time range (60 min). However, at 240 °C, a decrease starts to take place after reaching a maximum at 20 min (Stadler *et al*., 2002 a). This behavior is

consistent with the pattern reported by Casal *et al*. (2000 a), although they found a peak at 15 min.

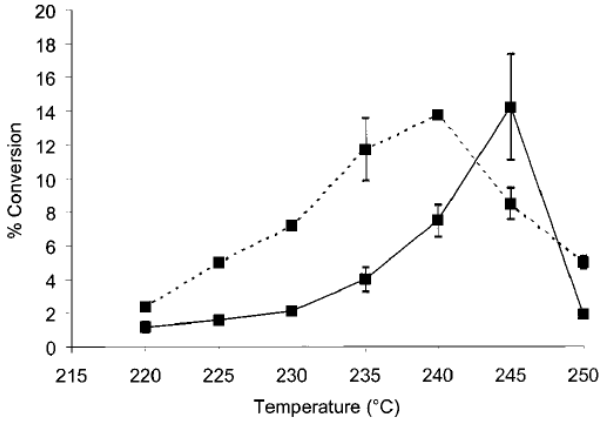


Figure 1.6. Formation of nicotinic acid (dashed line) and 1-methylpyridinium (solid line) from trigonelline hydrogen chloride as a function of temperature at a constant time (15 min). Values are expressed as percent of conversion on a molar basis (Stadler *et al.*, 2002 a).

Table 1.3. Formation of nicotinic acid from trigonelline hydrochloride as a function of temperature and time Expressed as percent conversion on a molar basis (adapted from Stadler *et al.*, 2002 a).

time (min)	220 °C	230 °C	240 °C
5	0.35	1.47	5.26
10	1.56	5.10	14.10
20	4.34	12.75	16.85
30	6.86	14.26	11.36
45	9.30	15.27	10.00
60	10.85	17.08	6.70

The significant increase of nicotinic acid during the first 20 min of roasting at 240^{o}C, as in the case of 1-methylpyridinium, is consistent with the degradation of trigonelline (**Table 1.1**), confirming its role as the major source of nicotinic acid (Stadler *et al*., 2002 a).

1.4.3. Caffeoylquinic Acids (CQAs)

Caffeoylquinic acids are an important group of phenolic metabolites produced by certain plant species like potatoes, tomatoes, apples, pears, and coffee, where they are found at particularly

high levels (Farah and Donalgelo, 2006; Lallemand *et al*., 2012). These compounds are formed through the reaction of quinic acid with caffeoyl-CoA, catalyzed by the quinate-*O*-hydroxycinnamoyltransferase enzyme (Dewick, 2009). Beside their relevance in plant physiology, caffeoylquinic acids have a number of beneficial health properties related to their potent antioxidant activity (Farah and Donangelo, 2006) and anticarcinogenic potential (Boettler, 2011).

Caffeoylquinic acids are considered as the main group of chlorogenic acids in green coffee, and in consequence, play an important role in the taste quality of the coffee brew (Moon *et al.*, 2009, Blumberg *et al*., 2010), contributing to the final acidity, astringency and bitterness of the beverage (Farah and Donangelo, 2006).

Among the various caffeoylquinic acids identified in coffee, the monocaffeoyl isomers, 3-, 4- and 5-CQA (structure **5** in **Figure 1.4**) are the most abundant and investigated compounds. There is an agreement in the literature regarding the order of abundance of these isomers, both in green and roasted coffee, 5-CQA being the most prominent compound, accounting for about 56 – 62 % of total chlorogenic acids (Farah and Donangelo, 2006), followed by 4-CQA and 3-CQA (Fujioka and Shibamoto, 2008; Moon *et al.*, 2009; Blumberg *et al.*, 2010) (see **Table 1.4**). However, during roasting, significant decreases of 5-CQA levels were reported (**Tables 1.4** and **1.5**), whilst the content of the 3-CQA and 4-CQA isomers were reduced in a lesser extent. As a consequence, under city and french roast conditions, the amount of 4-CQA and 5-CQA are almost the same (**Table 1.4**). Prolonged roasting periods and high temperatures are responsible for the almost total loss of these compounds due to their thermal inability. This behavior needs to be considered when defining the roasting conditions, given the pharmacological potential of this group of compounds.

Table 1.4. Monocaffeoylquinic acid content in Nicaraguan coffee beans roasted under different conditions (values are expressed in mg / g dry mass basis) (adapted from Moon *et al.*, 2009).

Origin	Green	light roast[a]	medium roast[b]	city roast[c]	french roast[d]
3-CQA	**3.52**	**4.65**	**3.50**	**0.50**	**0.09**
5-CQA	**40.19**	**12.22**	**8.19**	**1.08**	**0.21**
4-CQA	**5.96**	**7.12**	**4.90**	**0.87**	**0.17**

Roasting term conditions: [a] 12 min at 230 °C, [b] 14 min at 240 °C, [c] 17 min at 250 °C, [d] 21 min at 250 °C.

The contents of 5-caffeoylchlorogenic acid found in green coffee beans of different origins and under different roasting conditions are shown in **Table 1.4** (Moon *et al*., 2009). Very little variation in 5-CQA content, in terms of geographical origin, except in the case of the Ethiopian coffee, can be observed. As mentioned above, the amount of 5-CQA varies substantially with the intensity of roasting degree, reaching the lowest level under french roasting conditions in all cases. When comparing the behavior of 5-CQA during roasting in Ethiopian and Panamanian coffees, for example, it can be appreciated that matrix effects may also affect the changes in 5-CQA concentration, especially for the light and medium roasts.

Table 1.5. 5-CQA content in coffee from different origins and with different roasting degrees in mg / g dry mass basis (adapted from Moon *et al*., 2009).

Origin	Green	light roast[a]	medium roast[b]	city roast[c]	french roast[d]
Ethiopian	**50.70**	**15.11**	**9.27**	**0.79**	**0.16**
Nicaraguan	**40.19**	**12.22**	**8.19**	**1.08**	**0.21**
Panamanian	**40.15**	**15.88**	**11.04**	**1.17**	**0.23**
Sumatran	**41.65**	**14.74**	**10.11**	**1.57**	**0.30**

Roasting term conditions: [a] 12 min at 230 °C, [b] 14 min at 240 °C, [c] 17 min at 250 °C, [d] 21 min at 250 °C.

A consistent correlation between the pH of the solution and the degree of roasting was also noted by Moon *et al*. (2009). The pH increased from 5.7 to 6.2 through light to french roast, results which may suggest that caffeoylquinic acids are important contributors to the degree of acidity of the coffee brew.

1.4.4. 5-Hydroxymethylfurfural (5-HMF)

5-Hydroxymethylfurfural, structure **6** in **Figure 1.4**, is an aliphatic aldehyde product of the Maillard reaction, present in a wide range of thermal treated foodstuffs. It is formed during the carbohydrate degradation that leads to reactive fructofuranosyl cations (Perez-Locas and Yaylayan, 2008). It has been demonstrated that this compound produces hepatocarcinogenic activity in female mice after a 2-year administration of 188-375 mg / kg day (NTP, 2010), however, in mammalian cells, no significant genotoxic effects were observed (Perez-Locas and Yaylayan, 2008, Abraham *et al*., 2011). Based on these studies, a daily intake of 80 – 100 mg / kg body weight was established as the safety margin for its consumption (Abraham *et al*., 2011).

5-HMF is less volatile and chemically more stable, and is therefore more prone to accumulate and persist in food than are similar compounds (Nikolov and Yailayan, 2011). It has been recognized as an indicator of heat damage or excessive storage in various foodstuffs, and, in consequence, is being used nowadays as a deterioration marker in some food quality controls, including coffee (Charlton *et al.*, 2002; Perez-Locas and Yaylayan, 2008; del Campo *et al*., 2010). Recently, 5-HMF has been reported in a number of instant coffee samples in amounts that range from 1 to 6 mg / g of solid product (del Campo *et al*., 2010).

1.4.5. Low molecular mass organic acids

As mentioned previously, acidity is an important sensory quality of the coffee brew. It has been estimated that the acids present in coffee account for about 11 % of the green coffee bean mass and 6 % of the roasted coffee bean mass (Galli and Barbas, 2004). Various low molecular-mass organic compounds contribute to this feature. The structures of some of the major aliphatic acids present in green and roasted coffee beans are listed in **Figure 1.7**.

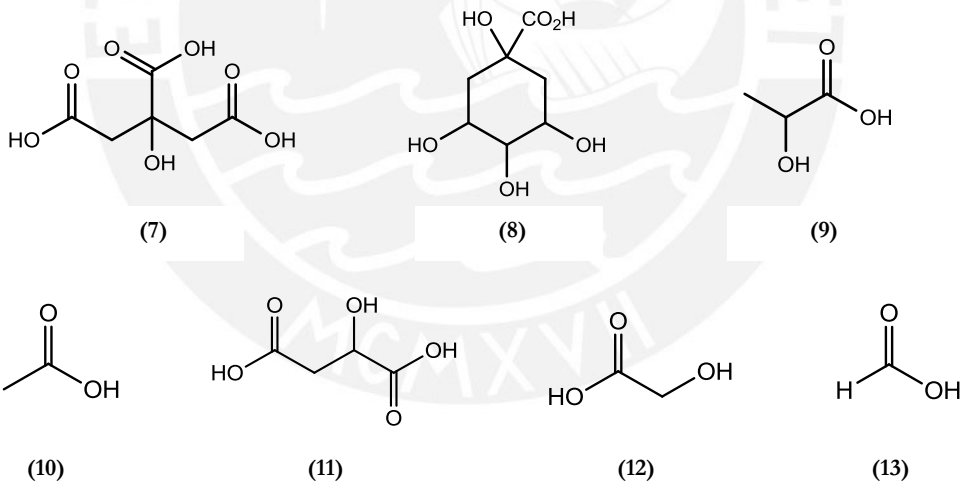


Figure 1.7. Chemical structures of the major organic acids found in roasted coffee: citric (7), quinic (8), lactic (9), acetic (10), malic (11), glycolic (12), formic (13).

Table 1.6 shows the variation in different acid contents as a function of organic roasting loss (ORL). As can be appreciated from this Table, the amount of some of these acids is greatly modified by the roasting process. As roasting progresses (higher ORL), the content of citric and malic acids decreased, whereas those of quinic, formic, acetic, glycolic and lactic acids increased.

Table 1.6. Acid content in g / kg as a function of organic roast loss (ORL%) (adapted from Clarke and Vitzthum, 2001).

Acid	0.0 % (Green)	3.3 %	4.2 %	5.1 %	6.4 %
Formic	nd	2.29	2.53	2.47	2.28
Malic	4.02	3.29	3.11	2.49	2.00
Citric	13.11	11.1	9.53	7.66	6.34
Glycolic	nd	1.28	1.64	1.81	2.02
Acetic	0.29	3.76	4.18	4.86	4.98
Lactic	nd	0.73	1.00	1.35	1.30
Quinic	6.87	9.96	8.81	8.94	9.12

[a] The conditions of roasting were not reported. The organic roast loss is proportional to the increase of roasting degree.

Quinic, malic and citric acids are the major organic acids, both in green and roasted coffee. The increase of quinic acid is a consequence of the chlorogenic acid cleavage, whereas citric and malic acids are degraded during the process to give other acidic compounds (Clarke and Vitzthum, 2001). Sucrose has been identified in model systems as the principal green bean precursor of formic, acetic, glycolic and lactic acid, whose contents are considered negligible in the green bean, but increase considerably during roasting (Ginz *et al*., 2000). A major fraction of the acidity can be attributed to these four aliphatic acids. **Figure 1.8** illustrates the formation of these compounds as a function of temperature, where it can be appreciated that formic and acetic acid began to fall off after reaching a maximum at 240 °C, whereas glycolic and lactic, which are less volatile acids, continued to increase. The decrease of these compounds, together with that of citric and malic acids, correlates with the lower acidity perceived in high-roasted coffees, where bitterness becomes the dominant sensory feature (Clarke and Vitzthum, 2001).

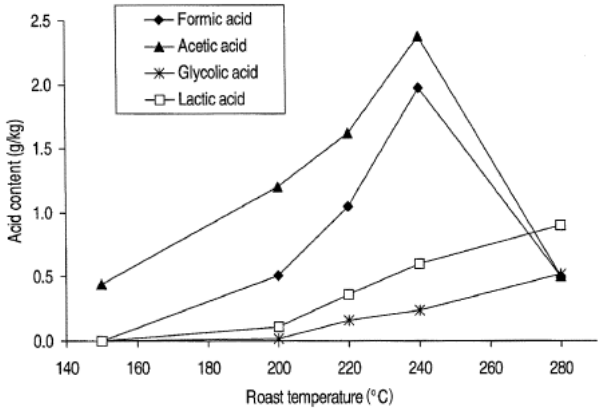


Figure 1.8. Acid content (g/kg) of formic, acetic, lactic and glycolic acids as a function of temperature (adapted from Clarke and Vitzthum, 2001).

1.5. Summary and Hypothesis

The relevance of the compounds previously discussed in the flavor and aroma profiles of coffee, in addition to the significant role they play during the roasting process, make these compounds suitable as chemical markers to monitor the roasting process.

As mentioned previously, this thesis is part of a project whose objective is to help over 2000 families, who constitute the association CENFROCAFE, to improve their roasting protocols. For this particular purpose, a fast and validated strategy is needed to quantify all these markers.

Of all the possible quantitative methods to be used for this purpose, quantitative nuclear magnetic resonance (q-NMR) seems an attractive approach for many reasons, although is not yet considered a standard method in food analysis. By NMR, sample preparation, solvent consumption, and the time of the analysis are diminished, when compared to chromatographic protocols, such as GC-MS, HPLC-DAD, and LC-MS. In addition, it affords, in only one experiment, information on all of the compounds present in the sample analyzed, data that can be directly used to identify and quantify the constituents of the food extract sample (Manina *et al*., 2012). A chemometric approach, employing Principal Component Analysis (PCA), will be also addressed in this study in order to distinguish differences in roasting degree.

Hence, the hypothesis of this work is that the new q-NMR methodology developed will allow, in a short-time experiment, distinctions to be made between different grades of coffee roasting by monitoring the markers previously identified (compounds **1 - 6**), and that these results will be in good agreement with classical and validated HPLC-DAD methodology. Additionally it is expected that this data will be useful to help set up the quality control protocol to be followed in order to optimize the roasting process performed by CENFROCAFE.

In the next chapter, the methodologies, q-NMR and HPLC-DAD and PCA, related to these coffee studies, are presented.

2. QUANTITATION STRATEGY OF SELECTED COMPOUNDS

Most of the studies previously discussed for the monitoring and quantitation of non-volatile small organic compounds in coffee are based in chromatographic techniques, and among them, HPLC (with different detectors MS, DAD and UV) was the most extensively applied (Casal *et al*., 2000 a,b; Stadler *et al*., 2002 a,b; Bastos and Alves, 2004; Farah *et al*., 2005; Alves *et al*., 2006; Clifford *et al*., 2006; Fujioka and Shibamoto, 2008; Moon *et al*., 2009). These techniques are time-consuming, involving the use of large amounts of organic solvents and require tedious sample pre-treatments. However, HPLC remains a recognized methodology to quantitate coffee (AOAC, 1995; AOAC, 2000).

In the last few years, Nuclear Magnetic Resonance (NMR) has shown enormous potential towards application in the field of food science in terms of foodstuff quality, geographical origin, variety differentiation, origin authentication, and raw material safety (Manina *et al*., 2012). The increasing interest in the application of NMR arises from the capacity of this technique to provide simultaneous access to qualitative and quantitative information. Among the NMR techniques available, two are the most extensively applied in quantitation analysis: ^{1}H-NMR and ^{13}C-NMR (Heikinnen *et al*., 2003; Manina *et al*., 2012). The principal difference between these techniques is the recording acquisition time. This is due to the difference in the natural abundance, magnetogyric ratio and relaxation mechanisms between ^{1}H and ^{13}C nuclei that are rather favorable for proton, making the ^{1}H-NMR technique the most suitable for fast (5 – 15 min) and accurate quantitative measurements. A q-^{13}C-NMR analysis could take several hours. In consequence, ^{1}H-NMR was the technique chosen in the present study for the quantitative study of chemical markers in coffee.

Both strategies, chromatography and nuclear magnetic resonance, will be described in this chapter, with a special emphasis on the parameters involved in the ^{1}H-NMR quantitative analysis.

2.1. Quantitative NMR analysis of coffee beans

2.1.1. NMR Acquisition parameters

Nuclear magnetic resonance represents the most versatile and extensively applied spectroscopic tool in modern chemical research with a wide range of applications in the basic and applied

sciences. As mentioned earlier, the increasing interest in the quantitative application of ^{1}H-NMR arises from the capacity of this technique to provide access to quantitative information due to the fact that the areas of the ^{1}H-NMR resonances are proportional to the relative number of nuclei that give rise to each signal. However, it is important to stress that this is only true under well-established experimental conditions. Hence, the level of accuracy of the quantitative measurements depends on the acquisition protocol employed. The principal aspects of quantitative acquisition will be explained below.

The transition between energy levels that give rise to the NMR signal is induced by the application of a radiofrequency which provides a magnetic field, perpendicular to the static field that oscillates at the Larmor frequency of the spins. According to the vector model, when a pulse of θ is applied to the spin system (B_1 in **Figure 2.1**) the bulk magnetization vector (z-axis) rotates towards the y-axis at a θ angle (Williams and King, 1990). A high power 90° pulse, closer to a 10 μs pulse width, is the most commonly applied for NMR measurements to provide a wide frequency range (able to excite all the resonances in the spectrum) and avoid off-resonance effects[2] (Claridge, 2009). Since, after a 90° pulse, all magnetization is placed in the transverse plane x – y, a maximum signal intensity is achieved and the sensitivity is optimized, a feature that is essential for quantitative purposes. However, to improve the S/N ratio it is required to perform successive scans and wait for the signals to fully relax, thus extending the recording periods.

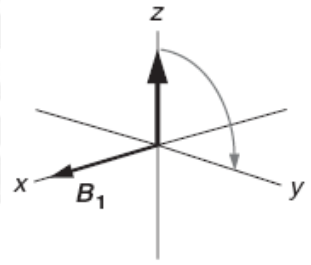


Figure 2.1. Vectorial representation of an rf pulse applied to a spin system (reproduced from Claridge, 2009).

A feature of major importance for the resolution and sensitivity of the method is the avoidance of differential saturation effects which arise from spins with different relaxation rates. A proper recording time needs to be established to guarantee that the acquired data actually reflect the relative contents of the different metabolites present in the sample (Pauli *et al*., 2005; Burton *et*

[2] Off-resonances effects arise when the rf excitation does not match precisely the Larmor Frequency giving rise to nuclei resonance outside the excitation bandwidth of the pulse.

al., 2005). Following rf pulse perturbation, nuclear spins of the sample re-establish equilibrium conditions by different relaxation mechanisms. There are two principal processes for spin relaxation: longitudinal (T_1) and transverse relaxation (T_2).

Longitudinal relaxation consists in the recovery of the +z magnetization after pulse excitation of the nuclear spins and hence in the equilibrium re-establishment of the system (**Figure 2.2**). This process follows an exponential behavior (**Figure 2.3**), determined by the relaxation time constant, T_1. As can be seen in **Figure 2.3** full recovery (~99.33%) is essentially achieved after a period of $5T_1$ (Burton *et al*. 2005; Claridge, 2009). Thus longitudinal relaxation time knowledge of the nuclei present in the sample is a parameter that has to be considered for quantitative NMR experiments.

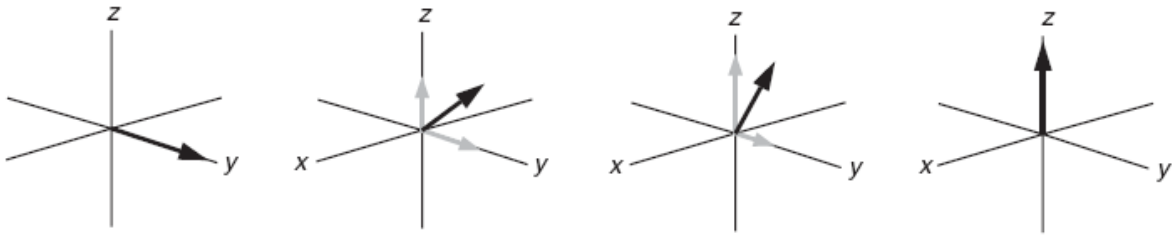


Figure 2.2. The recovery of the magnetization vector re-established the longitudinal component in z-axis (reproduced from Claridge, 2009).

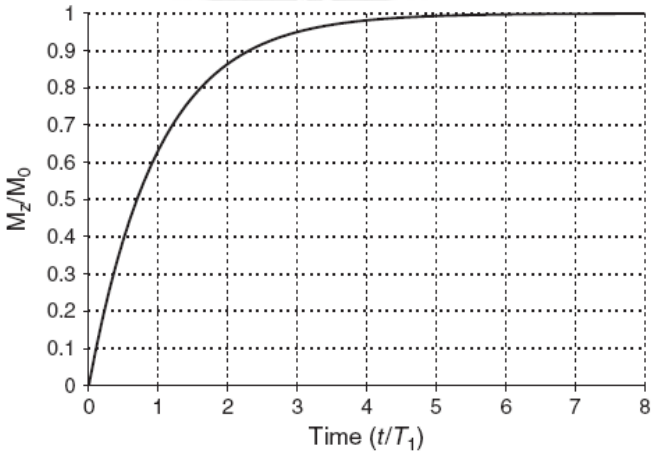


Figure 2.3. Evolution of the exponential growth of longitudinal magnetization (reproduced from Claridge, 2009).

Typical T_1values for hydrogen are in the range of 0.5 to 5 s for most medium-sized organic molecules (MW=100-500), whereas carbon T_1s may require minutes for full recovery. This is principally a consequence of dipolar relaxation (Claridge, 2009), the dominant relaxation mechanism for proton and carbon nuclei. According to this mechanism, the relative positions of two neighboring nuclei affects the local field experienced as a result of dipolar interactions and molecular tumbling in solution. Thus, ^{1}H and ^{13}C nuclei require the presence of neighboring protons as a source of magnetic dipoles to induce relaxation. The higher availability of this source of relaxation for ^{1}H nuclei, especially when compared to quaternary carbons, makes hydrogen T_1 values, and thus ^{1}H-NMR, more suitable for practical purposes than ^{13}C-NMR, leading to shorter recording times.

Transverse relaxation is a consequence of the local magnetization field experienced by each nucleus due to magnetic field inhomogeneity and principally to local magnetic fields arising from intramolecular and intermolecular interactions, which may result in no net magnetization in the transverse plane. As with longitudinal relaxation, transverse relaxation occurs with an exponential decay, now characterized by the time constant T_2. This parameter is inversely proportional to the widths of the NMR resonances, since a short T_2 corresponds to fast decaying FIDs, whereas longer FIDs give narrower resonances (**Figure 2.4**).

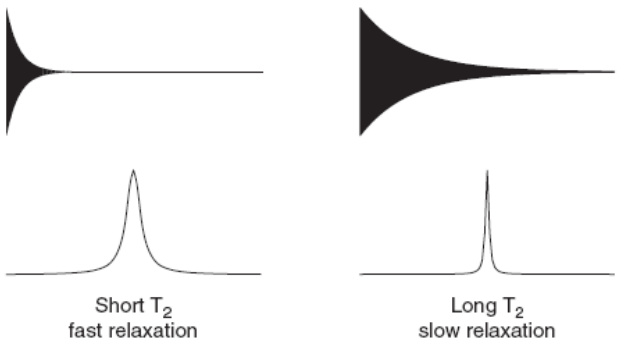


Figure 2.4. Transverse relaxation times for high and low molecular weight compounds (reproduced from Claridge, 2009).

Higher molecular weight molecules typically display shorter T_2 times than smaller ones, and in consequence give broader NMR resonances. If the difference is sufficiently large, the faster relaxing species can be selectively reduced in intensity with the Carr-Purcell-Meiboom-Gill (CPMG) echo sequence, allowing editing of the spectrum according to molecular size (Claridge, 2009; Manina *et al*., 2012). **Figure 2.5** shows a successful application of this approach where the

signals of camphor, a small molecule, can be appreciated after the T_2-filtering of the broad resonances of a heavy molecular weight polymer (Claridge, 2009).

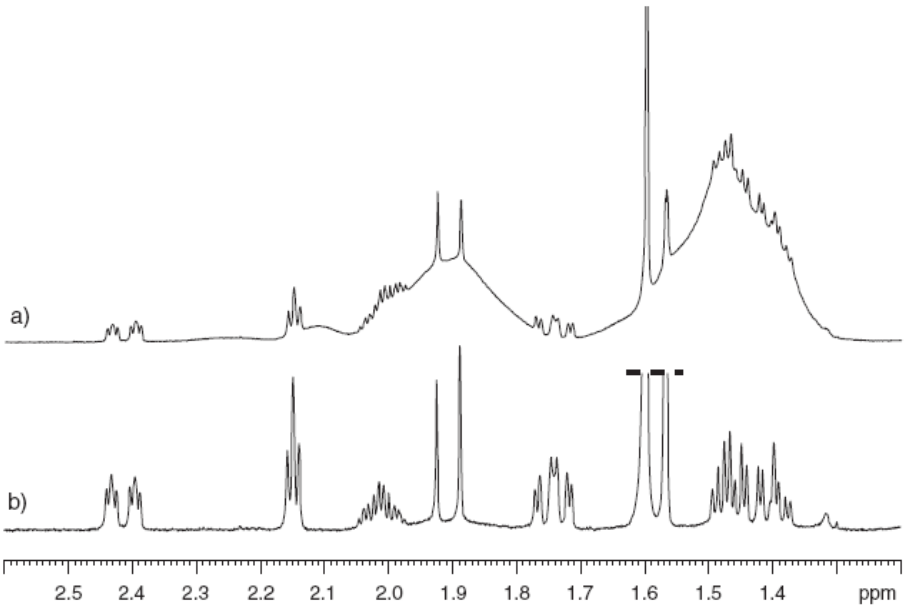


Figure 2.5. T_2 filter for a camphor sample contaminated with polysytrene (MW=50000). The broad resonances of the polymer in (a) have been suppressed in (b) through T_2 based-editing with the CPMG sequence (reproduced from Claridge, 2009).

Large solvent resonances may also affect the ^{1}H-NMR spectrum, principally by baseline distortion and masking of solute resonances due to severe broadening of the water signal, related to the loss of transverse magnetization (reduced apparent T_2) (Pauli *et al*., 2005; Claridge, 2009). Pre-saturation of the solvent is the most robust technique to reduce the magnitude of the solvent resonance before the NMR signal reaches the receiver. In the case of a 1D spectrum, the NOESY-presat sequence is the most commonly applied in qNMR analysis (Claridge, 2009, Manina *et al*., 2012), it involves the application of a non-selective 1D NOESY with short mixing time to assure solvent suppression. Pre-saturation of solvent signals could induce intermolecular nuclear Overhauser enhancements (NOEs) or cause partial saturation of signals from exchanging protons and in consequence, it might be considered as a potential source of error (Burton *et al*., 2005).

Like other quantitative methods, quantitative NMR measurements require calibration. Typically this process is achieved through the addition of an internal standard, a compound with a known concentration against which the area of the signals from the analytes will be compared. However,

although the internal standard method is considered the most accurate procedure (Pauli *et al*., 2012), it must satisfy some requirements that may not be fulfilled in complex matrices as food: it must be inert to the solute, its relaxation time should be similar to the analytes present in the sample, and its resonance signals should not overlap with those of the sample (Pauli *et al*., 2005).

Although it has not received much attention as the internal standard method, external standard calibration has also been validated for quantitative ^{1}H-NMR (Burton *et al*., 2005). In this case, spectra of standards and analytes need to be recorded under the same conditions to minimize error. The factors affecting the precision of the external standard method and their relative influence have been investigated and, according to this study, the principal error sources are the experimental temperature, deviation from 90° pulse, purity of calibration standards, pre-saturation method and integration (Burton *et al*., 2005).

Another alternative to the addition of a chemical internal standard is the use of a synthetic reference signal called ERETIC2. Electronic Reference to access in Vivo concentration (ERETIC2) is a new quantitation tool based on PULCON (Pulse Length based Concentration Determination) (Dreier and Wider, 2006) as an internal standard method that allows the correlation of the absolute intensities of two different spectra. This is possible because the lengths of a 90° or 360° pulse are inversely proportional to the NMR signal intensity. Therefore, if the samples are well calibrated, their concentration can be obtained from another sample, whose concentration is known precisely. For this experiment it is crucial to have well-defined quantitative acquisition parameters.

In addition to the parameters related to experimental acquisition, that were previously discussed, factors affecting the accuracy and precision of the data processing need to be considered, especially when dealing with complex spectra, given that these are potentially the most subjective aspects of NMR quantitation (Burton *et al*., 2005). Phasing and baseline correction are crucial, especially when baseline distortions are introduced by incomplete suppression of the solvent or the presence of impurities (Burton 2005). Subjectivity in the ranges of integration is inevitable in manual integration and is sample-dependent. Thus, the selection of sharp, non-overlapping and well-defined signals is critical for quantitation.

2.1.2. State of the art in NMR analysis of coffee

There are well-reported methods for the ^{1}H-NMR analysis of some fruit and vegetables, olive oils, alcoholic beverages, milk and dairy products, fish and meat, among others (Mannina *et al.*, 2012). However, there are only a few reported methods in the literature for the NMR quantitation of the metabolites in coffee, and most of them are based on ^{13}C-NMR spectra. Among these studies only one of them is validated by HPLC (del Campo *et al*., 2011), and other two correlate the qNMR results with the evolution of changes in chemical composition during roasting (Bosco *et al*., 1999; Wei *et al*., 2012).

Bosco *et al*. (1999) assessed for the first time the chemical changes in the roasting process of coffee by ^{1}H-NMR. They performed this study in two *C. arabica* and one *C. robusta* coffee samples, under three different degrees of roast. Both water (for hydrophilic molecule evaluation) and organic solvents (for hydrophobic and aromatic substance identification) were used for the coffee extract preparation. Significant differences among samples with different roasting degree were observed, including the decrease of trigonelline, chlorogenic acid, citric acid and formic acid content, and the increase of quinic acid levels.

More recently, Tavares and Ferreira (2006) investigated the structural elucidation of fourteen different commercial coffee samples by 1D and 2D NMR techniques. In this study *C. arabica* and *C. canephora,* were evaluated and nine important coffee metabolites including trigonelline, 1-methylpyridinium, formic acid, and quinic acid, among others were identified. Additionally they were able to quantitate caffeine in the complex coffee mixture employing a ^{1}H-NMR standard calibration curve.

The most detailed studies on the structural investigation of green and roasted coffee have been performed by the Wei research group (Wei *et al.*, 2010; Wei *et al.*, 2011). They were able to identify sixteen compounds in green coffee and almost thirty compounds in roasted coffee by means of 2D NMR techniques, such as edited heteronuclear single quantum correlation spectroscopy (HSQC), quantum filter correlation spectroscopy (DQF-COSY), constant time heteronuclear multiple bond correlation (CT-HMBC), rotating frame Overhauser effect spectroscopy (ROESY), among others. They also assessed the quantitation of several of these compounds by ^{13}C-NMR spectroscopy and studied the chemical composition evolution of some of them throughout the roasting process. However, despite the fact that they used a relaxing agent such as copper sulfate, the number of scans required in their measurements were of the order of 40000 which suggests extremely long and time-consuming experiments.

None of the studies previously mentioned include a validated quantitative method, such as HPLC, to corroborate their results. Recently, Del Campo *et al.* (2010) have performed a ^{1}H-NMR quantitative determination of important metabolites in various instant coffee brands that includes trigonelline, formic acid, caffeine and 5-hydroxymethylfurfural (5-HMF). To our knowledge this is the only study available in the literature that includes an HPLC validation of the ^{1}H-NMR quantitation results in coffee evaluation. Moreover, they found good agreement between the two techniques with percentage errors in the range of 1 to 20 %.

Although ^{13}C spectra show narrow and less overlapped compared with ^{1}H-NMR spectra, this technique has important disadvantages when compared to ^{1}H-NMR spectroscopy for quantitative purposes. In addition to the low natural abundance of the ^{13}C nuclei, these nuclei also have longer longitudinal relaxation times (several seconds or even minutes for quaternary carbons compared to a few seconds for proton nuclei) which results in longer acquisition times and in consequence, in time-consuming experiments. Even though the use of relaxing agents, containing paramagnetic species, such as chromium(III) acetylacetonate can reduce significantly the T_1s in ^{13}C experiments, it is the experience of this laboratory that this also hinders the sample preparation procedure (Maruenda and López, 2009).

Hence, the present study will address the quantitation of the selected metabolites **1** – **6** in coffee by the development of a new ^{1}H-NMR approach, for which all the parameters previously discussed: number of scans, receiver gain, relaxation time and pulse sequence, will be optimized.

2.2. HPLC-DAD quantitative analysis of coffee beans

As mentioned earlier, HPLC is the technique most extensively applied for the analysis of non-volatile foodstuffs, including the study of coffee. Several methods have been proposed for the selective analysis of specific compounds known to be relevant in the flavor and aroma profile of the coffee brew (Casal *et al*., 2000 a,b; Ky *et al.*, 2001; Yen *et al*., 2005; Alves *et al*., 2006). In **Table 2.1** the methods that have addressed the simultaneous quantitation of the compounds **1** – **6,** which are of interest for the present investigation, are listed. In addition, it includes the strategies for the separation of the chlorogenic acids, which are known to contribute significantly to the quality of the brew. It is important to mention that only one of them includes the determination of 1-methylpyridinium.

The method developed by Casal *et al*. (2000 a, b) achieved poor resolution for nicotinic acid and caffeine in the coffee sample, and the method of Ky *et al*. (2001) only achieved the separation of trigonelline and caffeine, which does not represent a difficulty due to their very different polarity.

The method developed by Yen *et al*. (2005) allowed the separation of trigonelline, nicotinic acid, 5-HMF, 5-CQA, caffeine and caffeic acid. However, this method involved the use of three different solvents for the system gradient and did not permit a good resolution or separation between trigonelline and nicotinic acid (**Figure 2.6**). All compounds in this study were monitored at only one wavelength, 280 nm.

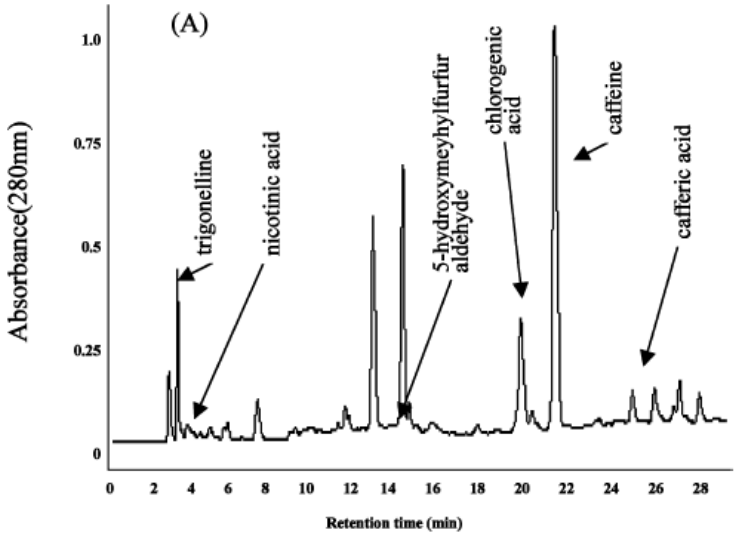


Figure 2.6. HPLC chromatogram of roasted coffee (reproduced from Yen *et al.*, 2005).

More recently, Alves *et al*. (2006) have reported a method for the simultaneous quantitation of nicotinic acid, trigonelline, 5-CQA, and caffeine in roasted coffee employing an acetic acid / acetonitrile mobile phase. They obtained good resolution employing ODS reverse phase columns, and the method achieved good recovery and repeatability values. This study did not include 1-methylpyridinium.

In addition to the difficulty in separating trigonelline from nicotinic acid, for which the methods available achieved only a slight separation (Δt), addressing the quantitation of 1-methylpyridinium ion in their presence represents a challenge. These compounds are very polar and share a pyridine skeleton, features that compromise selectivity. Another possible

complication is the cationic nature of 1-methylpyridinium that impedes its retention in C18 columns. Only one study (Stadler *et al*., 2002 b) has addressed its quantitation in coffee, but this method involved the use of an ion exchange column and did not include trigonelline determination. To the best of our knowledge only one, yet un-reported method (Maruenda *et al*., 2012) (**Table 2.1**), achieved the separation of trigonelline, nicotinic acid and 1-methylpyridinium with good resolution. This method includes the use of a Phenyl-hexyl Luna column (4.6 x 250 mm x 5µm) of dimensions similar to those reported by the other authors (**Table 2.1**). This column provides good selectivity for aromatic analytes due to interactions between the π electrons of the analyte and the π electrons of the phenyl ring bonded groups in the stationary phase. It is important to highlight that a method developed by Blumberg *et al.* (2010) also succeeded in the separation of several chlorogenic acids, including caffeoylquinic acid isomers 3, 4, and 5, by employing a Phenyl-hexyl Luna column (**Table 2.1**).

LC-MS will be used to corroborate the separation achieved by HPLC-DAD and to identify any new compound present in extracts of specialty coffee. Therefore the solvent-system required for the HPLC-DAD studies needs to be compatible with LC-MS. As seen in **Table 2.1**, most of the separation strategies presented used buffers that are not appropiate for LC-MS analysis, such as a phosphate buffer. Hence, for strategic reasons, associated with the CENFROCAFE-PUCP project (Maruenda *et al*., 2012), in which one of the objectives is to identify new quality markers in specialty coffee by LC-MS, the conditions that will be employed for the HPLC-DAD analysis will follow the protocol previously developed in this laboratory which are suitable for LC-MS analysis, the use of a phenyl-hexyl column using a formic acid / MeOH solvent.

In addition to the methodologies previously discussed, chemometric approaches are also being widely used in food science and traditional medicine, particularly in order to classify the data according to the origin, authentication and quality of the material (Mannina *et al*., 2012). In the following section, the suitability of one of these approaches, Principal Component Analysis, for coffee analysis will be discussed.

2.3. Chemometrics: Principal Component Analysis (PCA)

Chemometrics is generally defined as the application of statistical measurements to complex chemical and physical data with the aim of obtaining useful information as trends and patterns (Miller and Miller, 2010).

Table 2.1. Methods reported for the separation of compounds 1-6 in roasted coffee.

Author	Compounds studied	Technique	Column	Solvent	Wave lengths (nm)	T (°C)	Time (min)
Casal *et al.*, 2000 a, b	Caffeine, trigonelline, nicotinic acid	HPLC-DAD	Sprerisorb S5 ODS-1 (4.6 x 250 mm x 10 μm)	Phosphate (pH=4)/MeOH	264, 268, 276	n.k[a]	30
Ky *et al.*, 2001	Caffeine, trigonelline	HPLC-DAD	Sperpher 100 RP 18 (4 x 250 mm x 5 μm)	Et_3N-Acetic acid (pH=5.3)/MeOH	263, 272	n.k	15
Yen *et al.*, 2005	Caffeine, trigonelline, nicotinic acid, 5-CQA, 5-HMF, caffeic acid	HPLC-UV	Lichrosorb RP 18 (4.5 x 250 mm x 5 μm)	Ammonium dihydrogen phosphate (pH=2.6)/Phosphoric acid (pH=1.5)	280	25	30
Alves *et al.*, 2006	Caffeine, trigonelline, 5-CQA, nicotinic acid	HPLC-DAD	Sprerisorb ODS-1 (4.5 x 250 mm x 5 μm)	Acetic acid/MeCN	260, 272, 320	25	30
Blumberg *et al.*, 2010	Caffeoyl quinic acids (3, 4 and 5-CQA) and caffeoyl quinides	LC-MS	Phenyl-Hexyl Luna (4.5 x 250 mm x 5 μm)	Ammonium formate (pH=3.5)/MeOH	324	n.k	50
Maruenda *et al*, 2012	Caffeine, trigonelline, nicotinic acid, 1-methylpyridinium, 5-CQA	HPLC-DAD	Phenyl-Hexyl Luna (4.5 x 250 mm x 5 μm)	Formic acid (pH=2.6)/MeOH	260, 270, 330	30	40

[a] n.k = not known

Among the different chemometric techniques, Principal Component Analysis (PCA) is one of the most widely applied methods in the treatment of spectroscopic and chromatographic data obtained from NMR, HPLC and MS methodologies (Bruker, 2009; Manina *et al*., 2012) and is the method of choice for obtaining an overview of the data and identify clusters and outliers (Trygg *et al.*, 2007).

PCA can be defined as a projection method that converts multidimensional data into a low-dimensional model, usually with two or three dimensions simplifying its visualization and handling. The measure of the spread of a data matrix in each dimension or variable is given by the variance (**Eq. 1**), whereas the covariance (**Eq. 2**) determines how much of each dimension varies from the mean with respect to each other, to see if there is a relationship between them. Covariance is used by the PCA algorithm to find relationships between dimensions in the high dimensional data set. A positive value of covariance indicates that both dimensions increase and decrease together, a negative value indicates an inverse behavior between them and zero covariance indicates that the two dimensions vary independently.

$$var(X) = \frac{\sum_{i=1}^{n}(X_i-\bar{X})(X_i-\bar{X})}{n-1} \qquad \textbf{(Eq. 1)}$$

$$cov(X,Y) = \frac{\sum_{i=1}^{n}(X_i-\bar{X})(Y_i-\bar{Y})}{n-1} \qquad \textbf{(Eq. 2)}$$

To keep most of the variability, dimensions with constant values, low variance and linearly-dependent to other dimensions are ignored because they are not useful to explain the differences among the samples. In contrast, dimensions that do not depend on others, that have low covariance and high variance are kept (see **Eq. 1** and **Eq. 2**), so that the most important features can be extracted.

In order to focus on variation among the samples, it is necessary to translate the coordinate system to the center of the data cloud (**Figure 2.7**). This is done by subtracting the average from every column of variables of the data matrix (see **Scheme 2.1**). Changing the basis does not change the data, only its representation, with the aim to decrease the noise (low variance dimensions) and redundancy in the data set.

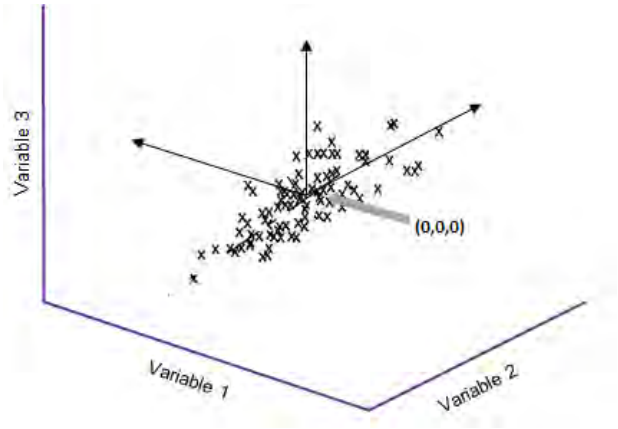


Figure 2.7. PCA centering through translation of the coordinate system (adapted from Trygg *et al.*, 2007).

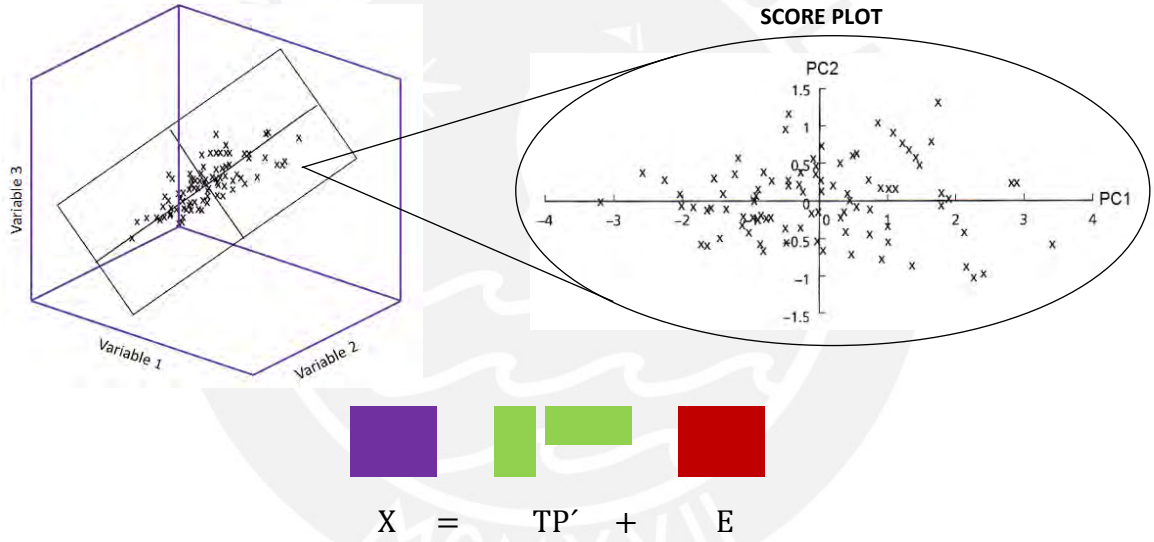
V_1	V_2	V_3				V'_1	V'_2	V'_3
2.2	5.0	2.1				-0.3	-0.4	-0.3
0.5	3.2	0.9				-2.0	-2.2	-1.5
2.9	7.5	2.2		$\bar{V}_1$ = 2.5		0.4	2.1	-0.2
4.8	8.5	4.0	→	$\bar{V}_2$ = 5.4	→	2.3	3.1	1.6
3.4	6.4	3.7		$\bar{V}_3$ = 2.4		0.9	1.0	1.3
0.9	2.4	1.1				-1.6	-3.0	-1.3
2.6	4.9	2.9				0.1	-0.5	0.5

Scheme 2.1. Simplified representation of the subtraction of the mean from dimensions in a three variable (V1, V2, V3) data set with seven samples (rows) so the new average of every column will now be zero (adapted from Farag *et al.*, 2009).

A schematic representation of the PCA treatment of a three dimensional data set is shown in **Scheme 2.2.** Each point in the system corresponds to different samples located in the three dimensional space as a result of the combination of the different variables. PCA first selects a normalized direction in the multidimensional space along which the variance is maximized, and then it finds a second direction along which the residual variance is maximized, however, because of the orthonormality condition, it restricts its search to directions perpendicular to the previous selected direction. These new set of axes are denominated as principal components and the resulting plane that best fits the data, a score plot (**T** in **Scheme 2.2**). The variances associated with each component quantify how principal or significant they are. As can be appreciated, most of the variation in the set of points can be described by this plane, i.e. a lower dimensional space

than the original. The residuals (**E** in **Scheme 2.2**) represent the distance between each point and its projection in the plane and correspond to the variability that is not explained by the model and is ignored because it is considered of lesser significance. The loading vectors (**P** in **Scheme 2.2**) define the relation among the variables and show their individual influence in the model. As directions in the loading plot correspond to directions in the score plot they are useful for identifying which variables or loadings separate different groups of objects (the scores). Thus, the loadings plots make possible the spectroscopic interpretation in chemical terms, of the statistical results.

PCA application can be extended to a bigger matrix **X**. If the dimensions are highly correlated there will be a small number of principal components with large variance enough to explain the data variability.



Scheme 2.2. Schematic representation of the principal component analysis (PCA) of three-dimensional data (adapted from Trygg *et al.*, 2007).

There are a few PCA approaches in the literature for the analysis of coffee employing NMR spectrometry. However, most of them are related to establishing geographical or variety differentiation (Wei *et al.*, 2012 b; Consonni *et al.*, 2012). An interesting PCA study was performed by Charlton *et al.* (2002) on instant coffee distinction according to the specific manufacturer, where 5-HMF was found as the best marker for this purpose.

PCA multivariate statistical analysis on two different roasted coffee varieties from various geographical origins has been performed by Casal *et al.* (2000 b) with data obtained from the

HPLC quantitation of trigonelline, nicotinic acid and caffeine. This study concluded that only trigonelline and caffeine could be useful for discrimination among different samples. More recently, Consonni *et al*. (2012) performed an ^{1}H-NMR-based PCA and PLS study also with the aim to develop a model for the geographical differentiation of *C. arabica* roasted coffees from three main production areas, Africa, Asia and America, which includes two Peruvian coffees. Their results indicate that fatty acids, chlorogenic acid, lactic acid, acetic acid and trigonelline where useful for sample separation according to geographical origin.

The PCA application developed by Wei *et al.* (2012 a) used ^{13}C-NMR data to study the evolution of chemical composition of the coffee bean during the roasting process. The PCA score plot resulting from these studies is shown in **Figure 2.8**, where a clear distribution of the coffee samples according to their roasting degree can be observed. This approach allowed them to identify chlorogenic acids, trigonelline and 1-methylpyridinium as chemical markers of the roasting process.

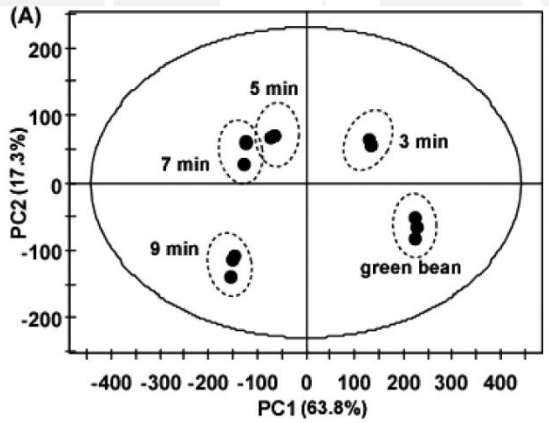


Figure 2.8. PCA score plot reported by Wei *et al.* (2012 a) from the ^{13}C-NMR spectra data of coffee samples with 3, 5, 7, and 9 min of roasting.

2.4. Summary of the strategy selected for the quantitation of compounds identified as chemical markers in roasted coffee

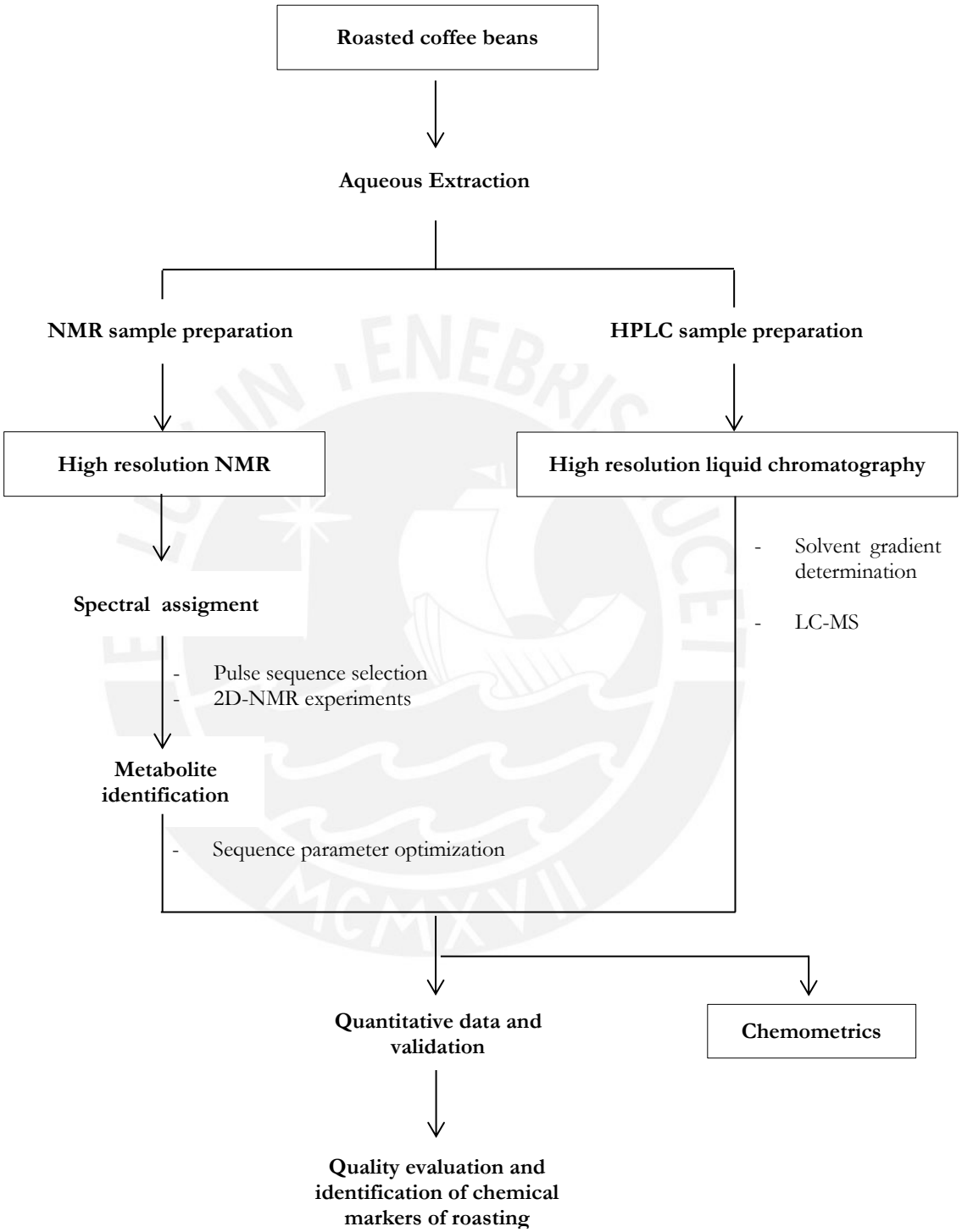
Scheme 2.3 summarizes the strategy that will be applied in the present study for the investigation of different Peruvian specialty roasted coffees. In the experimental section that follows, all the steps of the procedures will be described. The specialty coffee samples will vary in roasting degrees and in origin. An instant coffee sample is included for comparison purposes.

In general, the methodology selected for this study involves NMR, HPLC-DAD, LC-MS and PCA analysis, as shown in **Scheme 2.3**.

For the ^{1}H-NMR study, structural analysis (2D-NMR experiments) and pulse sequence selection (1D-CPMG and / or NOESY-Presat) will be addressed to determine the best acquisition parameters (T_1, RG, NS) for the identification and quantitation of the selected quality markers present in the coffee extract. This requires a specific NMR-sample preparation. The results obtained from the NMR experiments, identification of the major compounds present in the coffee extracts, will lead the HPLC-DAD study in terms of column, wavelength selection and elution gradients, in order to separate and quantitate the compounds of interest. The selective separation will be confirmed by LC-MS. In the case that the 1-methylpyridium ion is present in the coffee extracts under study, the use of a phenyl-hexyl-Luna C18 column will be required (Maruenda *et al*., 2012).

Finally, all of the data, qualitative and quantitative, will be analyzed by PCA.

Scheme 2.3. Strategy for the analysis of roasted coffee.



3. MATERIALS AND METHODS

3.1. Chemicals, materials and equipment

Chemicals: Pyridine (anhydrous, 99.8%), iodomethane and the standard compounds, trigonelline hydrochloride, caffeine, nicotinic acid and chlorogenic acid (5-caffeoylquinic acid, # 127K1640) were purchased from Sigma-Aldrich. 1-Methylpyridinium iodide was synthesized by Alonso Arguelles following a literature procedure (Son *et al.,* 2009). Oxalic acid was acquired from Mallinckrodt and di-sodium oxalate salt, from Merck. Methanol (HPLC-grade) was purchased from J.T. Baker, Co., and the water for HPLC and LC-MS analyses was MilliQ purified. Deuterated water and 3-trimethylsilyltetradeuteropropionic acid sodium salt (TSP) were purchased from Merck.

Materials: Five different roasted coffees were obtained from three different producers. APU Espresso, APU Gourmet and APU Classic were kindly supplied by CENFROCAFE-PERU (Jaen-Cajamarca, Peru) and differ from each other in quality and degree of roast. Two Huayabamba coffees with different roasting degrees (Espresso and House) were purchased in a local market. A commercial brand of instant coffee was also included in the study.

Equipment: All of the samples were lyophilized using a LABCONCO, Freeze Dry System / FreeZone 4.5.

One dimensional (^{1}H-NMR, ^{13}C-NMR) and two dimensional (COSY, HSQC, HMBC, TOCSY) NMR experiments were performed on a Bruker AVANCE - III 300 spectrometer using a broad band probe tuned to detect ^{1}H-NMR resonances at 300.13 MHz and ^{13}C resonances at 75.46 MHz. All spectra were acquired and processed using Bruker TopSpin^{TM} 2.1 software (Bruker BioSpin GmbH).

HPLC analyses was performed using an Agilent 1200 series-DAD equipped instrument with a binary pump unit (G1312A), UV-VIS detector (G1315D), degasser (G1379B), column oven (G1316A), and an auto-sampler (G1329B), controlled by Chemstation software LC3D (Agilent Technologies, Inc.). The column used was a Phenyl-hexyl Luna (250 x 4.6 mm, 5 µm, Phenomenex).

The LC-MS experiments were carried out on a Bruker Daltonics Esquire 6000 Ion-Trap mass spectrometer controlled by Compass 1.3 for Esquire / HCT software (Bruker Daltonik GmbH) with electrospray ionization (ESI). The HPLC system was the same as described for the HPLC - DAD.

3.2. Methodology

3.2.1. Extraction protocol for coffee samples

Twenty grams of roasted coffee bean samples were ground into a fine powder using an electric grinder (Sigma - Aldrich, model Z278181). Fourteen grams of the ground material were extracted, under constant stirring, with 40 mL of boiling water for 5 min in a closed plastic tube. The extract was quickly cooled in an ice bath for 15 min and then centrifuged (3500 x g) for 60 min at -10 °C. The turbid supernatant was lyophilized. Instant coffee was employed directly, without the extraction treatment. All coffee extracts were stored at -30°C after drying to preserve them for future analysis.

3.2.2. NMR analysis

3.2.2.1. Sample preparation

All samples, standards and dried coffee extracts (**3.2.1**), were treated according to a previously reported protocol (Son *et al.,* 2009), with minor modifications. Sixty milligrams of ground coffee were dissolved in 1 mL of 100 mM oxalate buffer (pH 4.0) and the solution was lyophilized until complete dryness (48 hours). The lyophilized material was re-dissolved in D_2O (1 mL) and lyophilized a second time (48 hours). Finally, the dry powder was taken into a final volume of 1 mL with D_2O and mixed with 100 μL of TSP-D_2O solution (3 mM). For quantitative NMR analysis, 600 μL of each sample was placed in a 5 mm diameter NMR tube.

3.2.2.2. NMR experiment

Deuterated water was used to lock the field frequency, and TSP was employed as internal reference. All samples were individually tuned and matched and data were collected at room temperature (~22 °C).

Pulse Sequence: An inter-pulse delay of 150 ms, and a T_2 value of 0.8 ms, were used for the 1D-CPMG (Carl Purcell Meiboom Gill) pulse sequence. The NOESY-Presat pulse sequence was applied to suppress the residual water signal. The transmitter frequency offset (O_1) was optimized for each sample.

Acquisition Parameters: The longitudinal relaxation time (T_1) was calculated using the inversion recovery NMR sequence. This value was optimized for the extract and the standard samples. Inversion delays used for recovery experiment were in the range of 0.5 to 60 s. The relaxation delay (D1) was set to five times the longest longitudinal relaxation time (5 x T_1). The number of scans was 32 and spectra were acquired with a receiver gain (RG) at a constant value of 90.5. A pre-scan delay of 10 μs was used. All other parameters were kept as set by the default. The time involved in the acquisition of each spectrum was 11 min. Two-dimensional experiments were performed with default parameters. All spectral data were collected at 64 K data points with a spectral width of 6188 Hz, using a 90° pulse length (9 μs pulse width).

Processing: Fourier transformation was performed with an exponential line broadening (LB) of 0.3 Hz for ^{1}H and 1.0 Hz for ^{13}C 1D NMR experiments. Phase and baseline correction were achieved manually, using Topspin 2.1 NMR software and ACD / NMR Processor 12.01 (Academic Edition).

3.2.2.3. ^{1}H-NMR standard calibration curves

Five different solutions containing standard compounds at various concentrations were prepared using D_2O as solvent. The concentration range, expressed in mg / mL for each compound, was as follows: 0.237 – 2.001 for trigonelline, 0.119 – 1.011 for 1-methylpyridinium iodide, 0.417 – 3.529, for caffeine, and 0.144 – 1.220 for 5-CQA. All solutions contained TSP-D_2O solution (3 mM).

The calibration curves were obtained by plotting the ratio between the peak area of the selected signal against that of TSP, with respect to the standard concentration in μg / mL. The linearity was checked by regression analysis of five different concentrations, and repeatability was assessed by two independent measures for each concentration, and five measures at a given concentration of each standard. The calibration curve was validated with an independent solution containing known amounts of each standard.

3.2.2.4. NMR analysis of coffee samples

Structural assignment: 1D and 2D-NMR spectroscopy (COSY and edited-HSQC) were used to assign the structures of the compounds present in the coffee aqueous extract, and in the mixture of standard compounds.

Quantitative methods: Reproducibility of the NMR data acquisition, and the processing parameters of real samples, were assessed by five repeat measurements of each extract. For this purpose, 60 mg of the dry coffee extract was treated as described in **3.2.2.1** and the mean peak area and standard deviation were calculated. Two different methods were employed to determine the concentration of selected compounds in coffee extracts: standard calibration curves (**3.2.2.3**) and ERETIC2. For ERETIC2 quantitation, a quinic acid / D_2O solution (1500 µg / mL) was employed as the reference sample for electronic calibration. For 90° pulse optimization, the pulse width was determined for three different signals, and was set to 1/4 of the 360° pulse length at which the signals are null (integral equal to 0) A 600 Hz width was employed. The determined value was employed to perform the NOESY-Presat sequence.

3.2.3. HPLC analyses

The mobile phase employed for separation was a mixture of two solvents: A (0.3% aqueous formic buffer, pH 2.4) and B (MeOH). The gradient elution program was: 0 - 10% B in 5 min, 10 - 25% B in 8 min, 25% for 3 min, 25 - 35% B in 6 min and 35 - 40% B in 8 min (total recording time = 30 min). The flow rate was 1 mL / min and the separation temperature was 30 °C. Nicotinic acid and 1-methylpyridinium were monitored at 260 nm, trigonelline and caffeine at 270 nm, and 5-CQA at 330 nm. The injection volume was 10 µL. All samples were filtered through a 45 µL syringe filter (Millipore, Germany) prior to analysis.

3.2.3.1. HPLC method validation

The HPLC method was validated according to ICH requirements (ICH, 1996). The external calibration curves were prepared with solutions containing standard compounds in the following ranges expressed in µg / mL: 27 - 217 for 1-methylpyridinium, 50 - 402 for trigonelline, 1.9 - 15 for nicotinic acid, 30 - 241 for 5-CQA, and 90 - 722 for caffeine. The linearity was checked by regression analysis of five different concentrations of each compound. Intraday and interday

precision were confirmed by performing injections on the same day (n=5) and over five different days, respectively, at a given concentration of each compound. Limit of detection (LOD) and limit of quantitation (LOQ) values were obtained from highly diluted solutions, assuming signal-to-noise ratios (S / N) of 3 for LOD and 10 for LOQ.

3.2.3.2. Qualitative analysis of aqueous extracts by LC-MS

The ESI - MS operating conditions for the positive ionization mode were: drying gas (N_2) flow, 12 L / min; nebulizer pressure, 65 psi; gas drying temperature, 350 ^{o}C; capillary voltage 4000 V, scan mode m/z 50 – 500; injection volume, 10 µL. For the negative ionization mode, conditions were as described for the positive mode, but with a voltage of 3800V. The elution program was the same as that employed for HPLC analysis.

3.2.3.3. HPLC analysis of coffee samples

For quantitative analysis, 12 mg of each coffee extract (**3.2.1**) was diluted with MilliQ water to a final volume of 1 mL and then filtered through a 0.45 µm syringe filter (Millipore, Germany) and injected in to the HPLC as described in **3.2.3.** Each sample was evaluated in triplicate. Quantitative determination of selected compounds was achieved from the calibration graphs (**3.2.3.1**).

3.2.4. Principal Component Analysis (PCA)

3.2.4.1. PCA of ^{1}H-NMR results

^{1}H-NMR spectral data of coffee extracts were reduced into 0.002 ppm spectral buckets employing a simple rectangular bucket method with AMIX Software Viewer 3.9, 2009 (Bruker BioSpin). The water suppressed region (4.7 to 5 ppm) was excluded, and the reference regions, left and right, were set at 10 and 0.3 ppm, respectively. The integration mode of intensities option was set as the sum of intensities, and the one used for scaling was scaled to maximum intensity. The resulting bucket tables were then imported to MATLAB 7.11 software (Matworks, Inc., Natick, MA) for further multivariate statistical analysis. Prior to PCA, all spectra were aligned by the Icoshift tool (Savorani, *et al*., 2010) and then scaled using Pareto scaling. A confidence ellipse in the score plots defined the 95% confidence interval of the model.

3.2.4.2. PCA of HPLC results

Principal component analysis of the HPLC results was performed employing a table elaborated on Microsoft Excel (Microsoft Office, 2010), containing information on the concentration of the evaluated standards for each coffee extract, calculated from the standard calibration graphs. The data was processed with MATLAB 7.11 software (Matworks, Inc., Natick, MA) as described in **3.2.4.1**.

4. RESULTS AND DISCUSSION

Six types of commercial coffee brands were selected for the present study, five of which are considered specialty coffees. This denomination is associated with the privileged environmental conditions where they grow, in terms of altitude, microclimate and soil. **Table 4.1** summarizes the principal characteristics of these five Peruvian specialty coffees. The sixth coffee type was a commercial instant coffee brand, selected for comparison purposes.

Table 4.1. Characteristics of the Peruvian specialty coffees under study.

Coffee brand	Location	*Coffea* species	Growing altitude (m.a.s.l.)	Cup score	Roasting time
APU Gourmet[a]	**Cajamarca**	***C. arabica***	**>1600**	**88-90**	**t1**[b]
APU Classic[a]	**Cajamarca**	***C. arabica***	**1200-1600**	**86-87**	**t1**[b]
APU Espresso[a]	**Cajamarca**	***C. arabica***	**1200-1600**	**86-87**	**t1**[b] **+ 2 min**
Huayabamba coffee a	**Amazonas**	***C. arabica***	**1400-2100**	**n.k**[c]	**t2**[b]
Huayabamba coffee b	**Amazonas**	***C. arabica***	**1400-2100**	**n.k**[c]	**t2**[b] **+ 2 min**

[a] APU brand provided by CENFROCAFE, Jaen-Peru; [b] proprietary information; [c] n.k = not known

The difference between APU Gourmet and APU Classic / APU Espresso is based on the different growing altitudes, whereas APU Classic and APU Espresso differ in roasting time, as do Huayabamba coffees, a and b.

The cup score values in **Table 4.1** are the result of the sensory tests (cupping) performed by the CENFROCAFE team. These tests evaluate specific flavor attributes, including fragrance/aroma, acidity, sweetness, body, aftertaste and balance. According to the Specialty Coffee Association of America (SCAA, 2009), a final scoring greater than 80 is considered a specialty coffee.

Taking into consideration that the altitude and the roasting conditions do affect the quality of coffee (Farah and Donangelo, 2006; Neves *et al*., 2012), this work is centered on a chemical analysis that will allow differentiation among all of the six coffee samples in terms of grades of coffee roasting and quality by monitoring certain chemical markers. The approach followed utilizes a HPLC validated methodology to support a non-classical, nuclear magnetic resonance (NMR) spectroscopy-based quantitative method.

Hence, the selection of an appropriate coffee extraction protocol, followed by the implementation of quantitative NMR methodology (optimization of NMR parameters) was required for the chemical analysis proposed.

4.1. Extraction protocol

All of the samples were ground and extracted with hot water (14 g / 40 mL) at 95 °C for 5 min, after which centrifugation (rpm 3500 g; T = -10 °C; t = 60 min) was used to separate the solid material from the aqueous extract. The results obtained for the five samples are shown in **Table 4.2**. It is clear from the Table that the extracted volume (14 - 16 mL) and the dried coffee extract mass (1.017 - 1.356 g) are comparable for all of the samples.

Table 4.2. Final volume and mass values of coffee extracts for the developed extraction protocol.

Coffee brand	Supernatant volume (mL)	Dried aqueous extract mass (g)
APU Gourmet	14.00	1.017
APU Classic	14.50	1.094
APU Espresso	16.00	1.121
Huayabamba coffee a	15.50	1.356
Huayabamba coffee b	15.90	1.247

When a different extraction protocol was tried, using a domestic espresso coffee maker, at a constant extraction time (20 s), and having ground identically an exact weight of coffee beans, the final volume of the coffee extract varied dramatically (15 – 30 mL). These erratic results may be due to the inability to control the tamper pressure in this non-professional coffee maker. This method was therefore not further considered in developing an extraction protocol.

4.2. Quantitative determination by NMR

4.2.1. NMR sample preparation

In any NMR sample preparation, due to the low sensitivity of this spectroscopic method, concentration is a parameter that must be defined early in the study. Highly diluted samples will produce signals with a low S/N ratio. On the other hand, samples which are highly concentrated

will induce signal broadening, which affects the resolution and makes structure determination more difficult (Mannina *et al.*, 2012). The concentration needs to be optimized, and in consequence, the solvent used to prepare the NMR sample is critical. Given that coffee extracts have a good solubility in water, D_2O was employed for the NMR analysis. The optimal sample concentration was set to 60 mg / mL of D_2O.

Considering that many of the coffee constituents are organic acids, the pH of the NMR solution needs to be controlled to minimize chemical shift variation. According to a literature review (Mannina *et al.*, 2012), two different buffers are commonly used for NMR food sample preparation: a phosphate buffer, which stabilizes pH in the range 6 to 7, and an oxalate buffer, for a pH = 4. Hence, due to the nature of the various organic acids involved (chlorogenic, citric, formic, quinic, among others), oxalate buffer was chosen for the study performed here.

In summary, as described in **section 3.2.2.1**, 60 mg of the lyophilized coffee extract was suspended in 1 mL of oxalate buffer. The lyophilized sample was re-dissolved in D_2O and then lyophilized for a second time. This protocol assures the maximum proton-deuterium exchange while keeping the pH constant. As shown in **Figure 4.1**, a sample not treated with buffer, and not lyophilized with D_2O (A in **Figure 4.1**), shows lower resolution than an oxalate - D_2O treated sample (B in **Figure 4.1**).

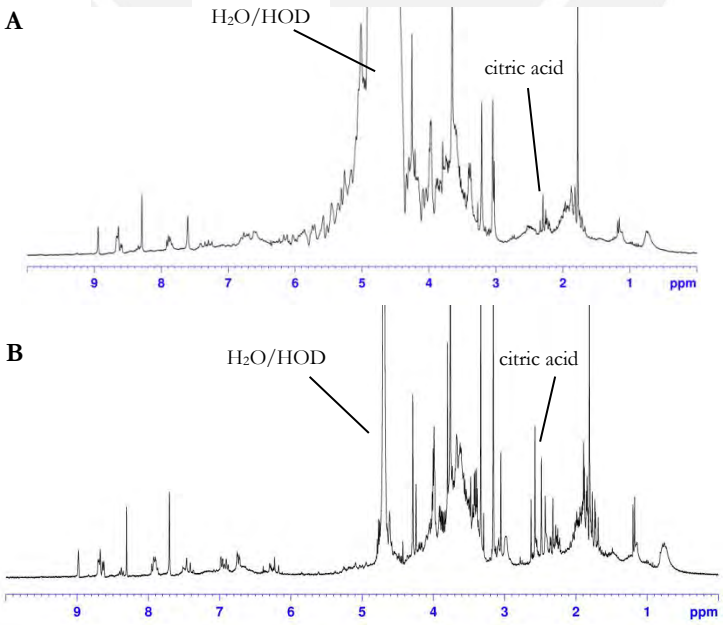


Figure 4.1. Typical ^{1}H-NMR spectrum of a coffee sample without (A) and with (B) oxalate-D_2O - sample treatment.

4.2.2. Spectral assignment of metabolites in roasted coffee samples

All structural assignments within the coffee extract spectra were verified by two-dimensional NMR techniques, such as COSY and edited-HSQC. The results were corroborated with the available literature for some of the compounds (Wei *et al.*, 2011; Wei *et al.*, 2012). This approach allowed for the simultaneous recognition of eleven compounds known to be associated with the flavor and aroma of coffee in the aqueous extracts (Casal *et al*., 2000 a,b, Clarke and Vitzthum, 2001; Stadler *et al*., 2002 a,b). Other spinsystems were also detected, but could not be assigned. Detailed information regarding the structures and the signal assignments is available in **Figure 4.2** and **Tables 4.3** and **4.4.**

It is clear from **Figure 4.3** that the region between 1 and 5 ppm is very complex due to the strong overlap between the signals from various metabolites in the matrix, and also to signal broadening. The latter may be due to the presence of very high molecular weight compounds, such as melanoidins, derived from the Maillard reaction (Borreli *et al.*, 2002; Wei *et al.*, 2011), polysaccharides and proteins. The presence of these macromolecules complicates the spectral assignment of signals in this region. Despite this, seven compounds were identified in this region of the NMR spectrum (**Figure 4.3**).

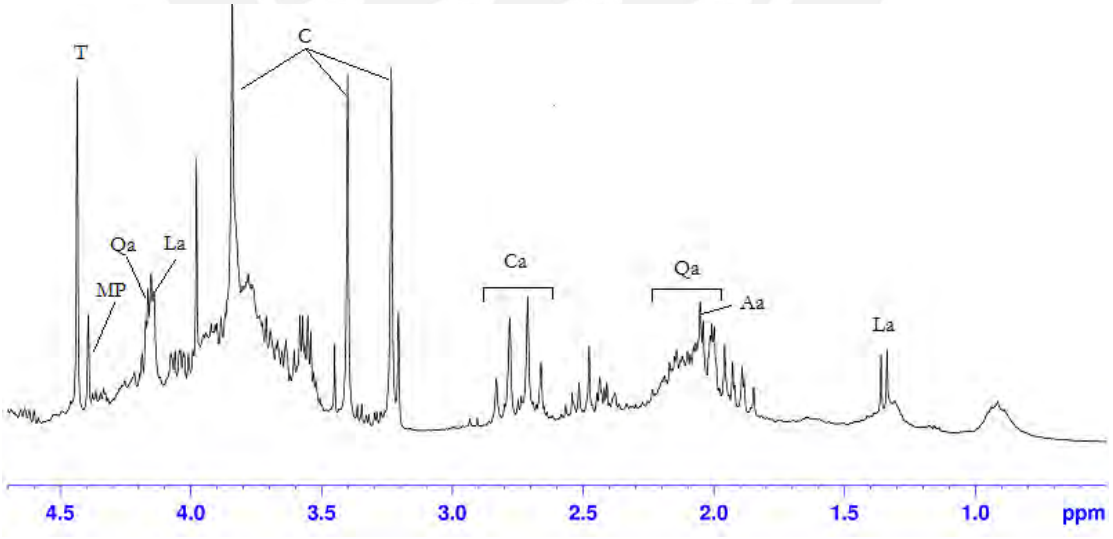


Figure 4.3. Expanded ^{1}H-NMR region (1 - 5 ppm) of a representative coffee extract under study. Compounds identified: T (trigonelline), MP (1-methylpyridinium), Qa (quinic acid), La (lactic acid), C (caffeine), Ca (citric acid), Aa (acetic acid).

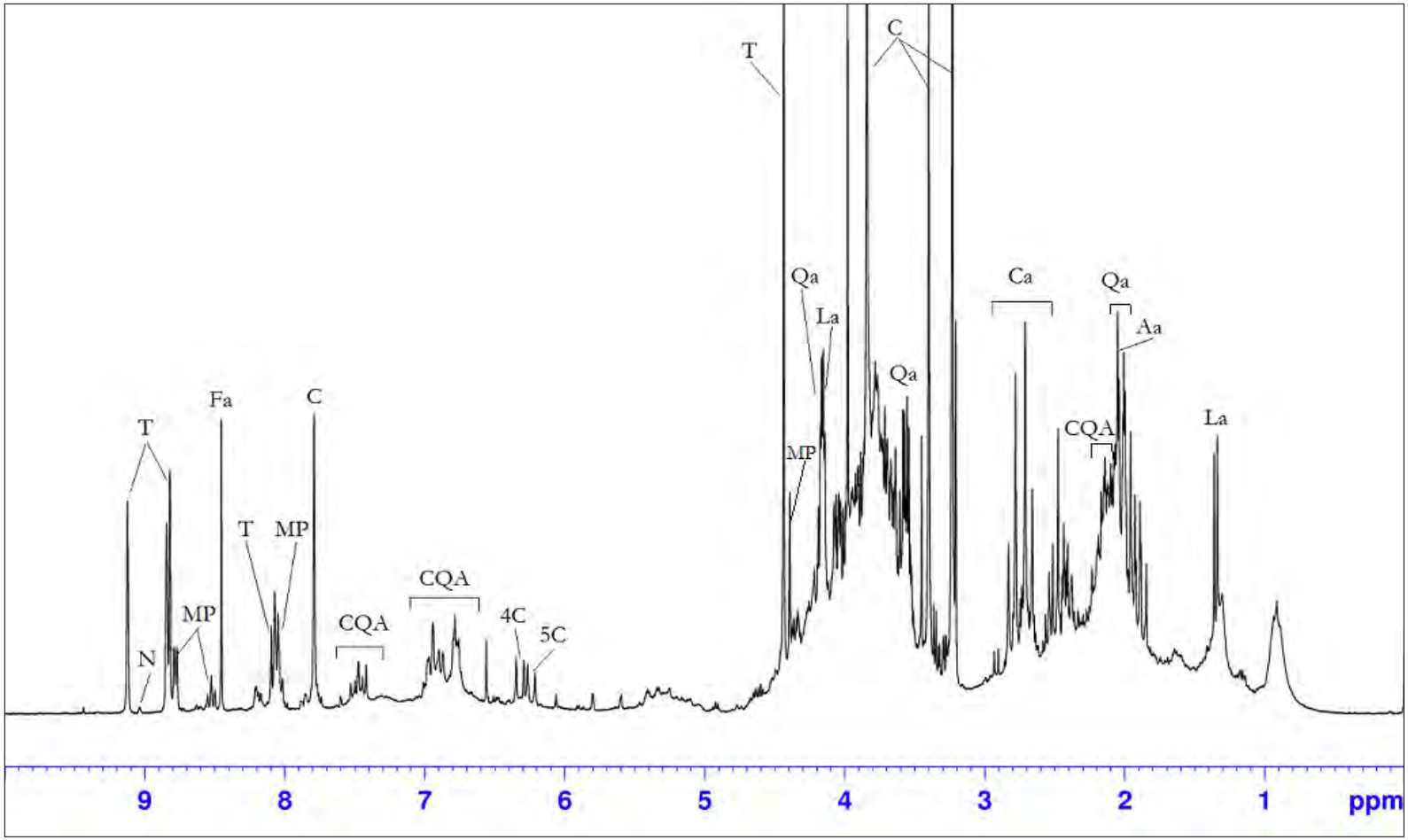


Figure 4.2. ^{1}H-NMR spectrum of a representative coffee extract sample. Compounds identified: T (trigonelline), N (nicotinic acid), MP (1-methylpyridinium), Fa (formic acid), C (caffeine), CQA (caffeoylquinic acid), 4C (4-caffeoylquinic acid), 5C (5-caffeoylquinic acid), Qa (quinic acid), La (lactic acid), Ca (citric acid), Aa (acetic acid).

Table 4.3. ^{1}H-NMR assignments of standard compounds identified in the coffee studied.

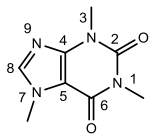
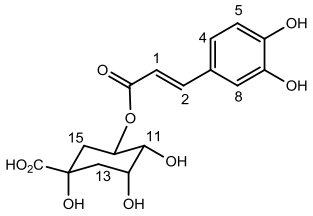
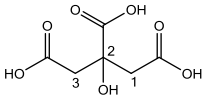
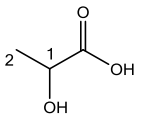
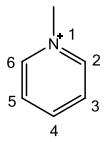
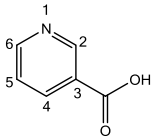
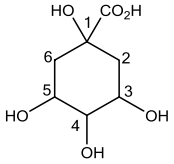
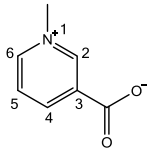
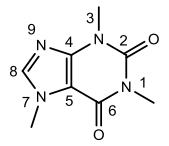
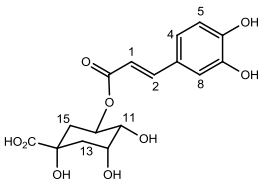
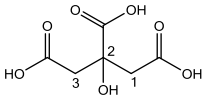
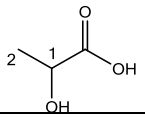
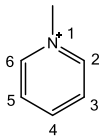
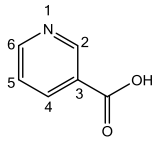
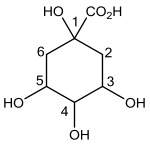
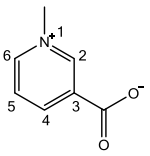
Metabolite	Assigment	δ_H (multiplicity)	Structure
Acetic acid (Aa)	**H-2**	**2.30 (s)**	CH_3CO_2H
Caffeine (C)	**H-8**	**8.09 (s)**	
	N7-CH_3	**3.77 (s)**	
	N1-CH_3	**3.32 (s)**	
	N3-CH_3	**3.15 (s)**	
5-Caffeoylquinic acid (5C)	**H-2**	**7.42 (d, 16.0 Hz)**	
	H-8	**7.01 (d 3.0 Hz)**	
	H-4	**6.94 (dd 6.0, 3.0 Hz)**	
	H-5	**6.78 (d, 6.0 Hz)**	
	H-1	**6.17 (d, 15.0 Hz)**	
	H-10	**5.15 (m)**	
	H-12	**4.12 (m)**	
	H-11	**3.75 (dd 9.0, 3.0Hz)**	
	H-15, H-13	**2.06 (m)**	
Citric acid (Ca)	**H-1a, H-3a**	**2.90 (d, 15.6 Hz)**	
	H-1b, H-3b	**2,72 (d, 15.6 Hz)**	
Formic acid (Fa)	**H-1**	**7.74 (s)**	HCO_2H
Lactic acid (La)	**H-1**	**4.24 (q, 12.0, 6.0 Hz)**	
	H-2	**1.28 (d, 6.0 Hz)**	
1-Methylpyridinium (MP)	**H-2/H-6**	**8.67 (d, 6.2 Hz)**	
	H-4	**8.41 (t, 6.9 Hz)**	
	H-3/H-5	**8.02 (t, 6.9 Hz)**	
	N-CH_3	**4.33 (s)**	
Nicotinic acid (N)	**H-2**	**9.12 (s)**	
	H-6	**8.88 (dt, 6.0 3.0 Hz)**	
	H-4	**8.31 (d, Hz)**	
	H-5	**7.58 (t, Hz)**	
Quinic acid (Qa)	**H-5**	**4.03 (q, 3.5 Hz)**	
	H-3	**3.91 (m)**	
	H-4	**3.42 (dd, 4.6, 3.4 Hz)**	
	H-2a/H-6a,b	**1.96 (m)**	
	H-2b	**1.79 (dd, 13.4, 11.0 Hz)**	
Trigonelline (T)	**H-2**	**9.21 (s)**	
	H-4, H-6	**8.82 (m)**	
	H-5	**8.02 (dd, 6.4 Hz)**	
	N-CH_3	**4.33 (s)**	

Table 4.4. ^{1}H-NMR assignments of compounds in the aqueous coffee extracts.

Metabolite	Assigment	δ_H (multiplicity)	Structure
Acetic acid (Aa)	**H-2**	**2.29 (s)**	CH_3CO_2H
Caffeine (C)	**H-8**	**7.79 (s)**	
	N7-H	**3.85 (s)**	
	N1-H	**3.40 (s)**	
	N3-H	**3.24 (s)**	
5-Caffeoylquinic acid (5C) (Wei *et al.*, 2010)	**H-2**	**7.47 (d, 16.0 Hz)**	
	H-5	**6.92 (d, 6.0 Hz)**	
	H-1	**6.25 (d, 16.0 Hz)**	
Citric acid (Ca)	**H-1a, H-3a**	**2.81 (d, 15.61 Hz)**	
	H-1b, H-3b	**2.68 (d, 15.61 Hz)**	
Formic acid (Fa)	**H-1**	**8.45 (s)**	HCO_2H
Lactic acid (La)	**H-1**	**4.15 (b)**	
	H-2	**1.35 (d, 6.98 Hz)**	
1-Methylpyridinium (MP)	**H-2/H-6**	**8.78 (d, 6.19 Hz)**	
	H-5/H-3	**8.02 (b)**	
	H-4	**8.53 (t, 7.75 Hz)**	
	N-CH	**4.39 (s)**	
Nicotinic acid (N)	**H-2**	**9.06 (s)**	
	H-6	**8.63 (d, 8.20 Hz)**	
Quinic acid (Qa)	**H-5**	**4.15 (m)**	
	H-3	**4.06 (m)**	
	H-4	**3.55 (dd, 9.32, 3.25 Hz)**	
	H-2a/H-6a,b	**2.05 (m)**	
	H-2b	**1.88 (dd, 10.64, 11.2 Hz)**	
Trigonelline (T)	**H-2**	**9.12 (s)**	
	H-4, 6	**8.83 (d, 7.2 Hz)**	
	H-5	**8.09 (t, 7.06 Hz)**	
	N-CH_3	**4.44 (s)**	

Signals pertaining to lactic acid, acetic acid and quinic acid were identified in the region from 1 to 2 ppm. The methyl group (H-2) of lactic acid (La) shows a distinctive doublet at 1.35 ppm. This signal overlaps with a broad signal which could not yet be assigned, and it correlates in the COSY spectra (**Figure 4.4**) to the H-1 multiplet signal at 4.15 ppm (orange circle, **Figure 4.4**). Both CH and CH_3 are confirmed as blue cross peaks in the HSQC spectra (**Figure 4.5**). Acetic acid (Aa) was identified by spiking through its distinctive singlet at 2.29 ppm, a signal that decreased substantially after the lyophilization treatment, as shown in **Figure 4.6**.

After a meticulous *J*-value analysis, the methylene groups of quinic acid (Qa) were assigned to the multiplets at 1.88 and 2.05 ppm (H-2, H-6), and associated with red cross-peaks in the HSQC spectrum (green circle in **Figure 4.5**). These protons correlate in the COSY spectra to both the multiplet at 4.15 ppm (H-5) and the doublet of doublets at 4.06 ppm (H-3) (red circle in **Figure 4.4**), and both signals were associated with the blue cross-peaks in the HSQC spectra (red circles in **Figure 4.5**). The doublet of doublets for H-3, in turn, correlates with the multiplet at 3.55 ppm (H-4) (red circle in **Figure 4.4**).

Citric acid (Ca) was identified by its characteristic and well-defined doublet of doublets between 2.7 – 2.8 ppm, which corresponds to the two equivalent methylene groups (H_2-1, H_2-3) adjacent to the carboxylic acid groups. The methylene identity of the signal was confirmed by the red cross-peaks in the HSQC spectra (green circle in **Figure 4.5**) and by *J* value comparisons against a citric acid standard sample (15.6 Hz). It is important to highlight at this point that the sample treatment developed for this study has allowed, at 300 MHz, a well-defined and resolved citric acid signal whereas previous results by Wei *et al.* (2011), using a higher field magnet (500 MHz), and with the sample under phosphate buffer conditions, resulted in broad signals which were attributed to the presence of metal-citrate complexes.

The methyl groups of caffeine, trigonelline and the 1-methylpyridinium ion (**Figure 4.3**), covalently bound to a heteroatom, were identified in the region from 1 to 5 ppm: singlets at 3.86 (N7-H), 3.43 (N1-H) and 3.26 (N3-H) ppm for caffeine, at 4.43 ppm (N1-H) for trigonelline, and at 4.39 ppm (N1-H) for the 1-methylpyridinium cation. All of these signals show blue cross-peaks in the HSQC spectra (purple circles in **Figure 4.5**). HSQC also allows for the assignment of the carbons directly bonded to these protons. The ^{13}C-NMR signal assignments for this region are noted in **Figure 4.5.** The assignment of these protons was further confirmed by their correlation to adjacent aromatic protons using the COSY spectrum (blue, purple and black circles, respectively, in **Figure 4.4)**.

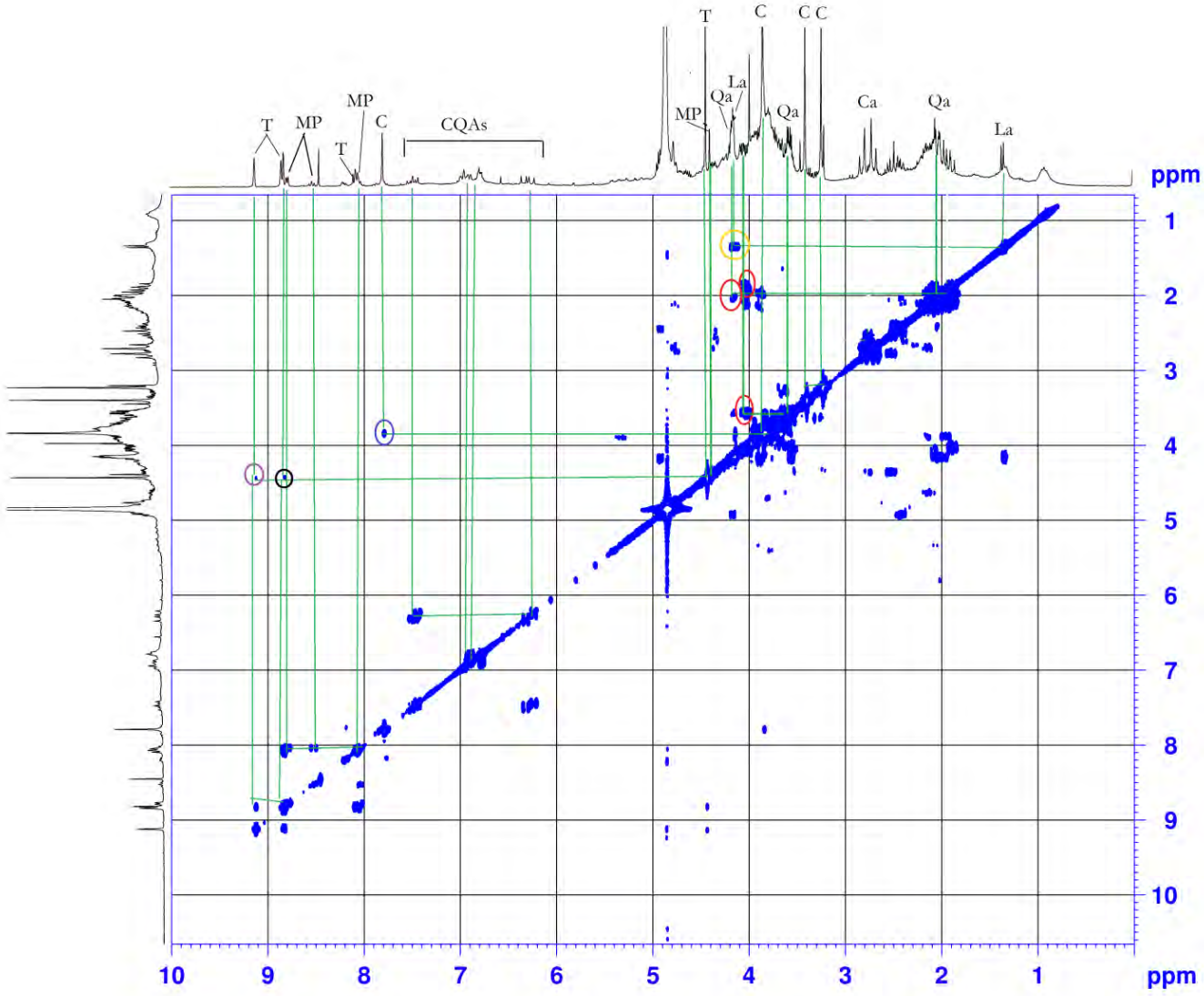


Figure 4.4. COSY spectrum of a representative roasted coffee extract under study.

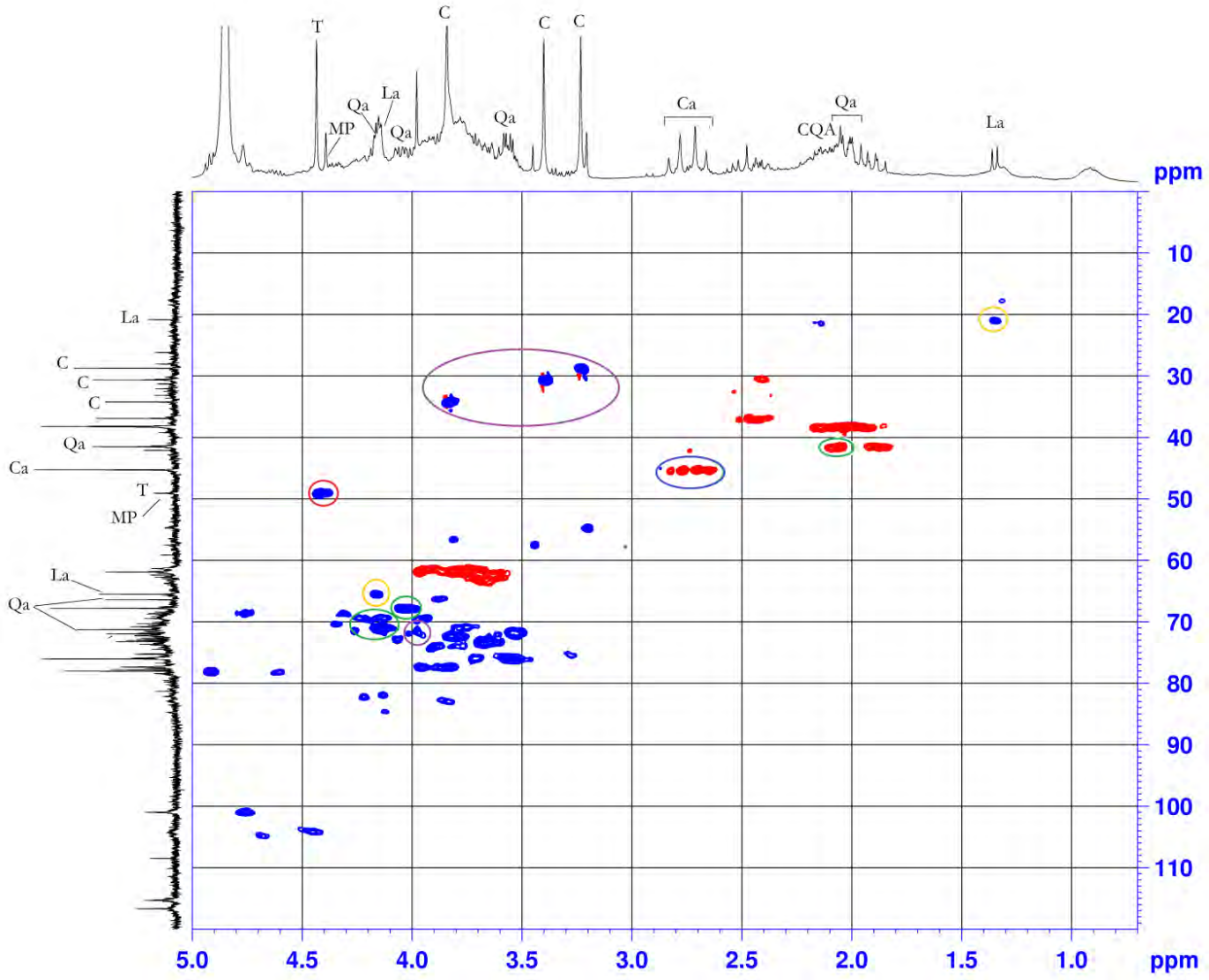


Figure 4.5. High field region of the HSQC spectrum of a representative roasted coffee extract under study.

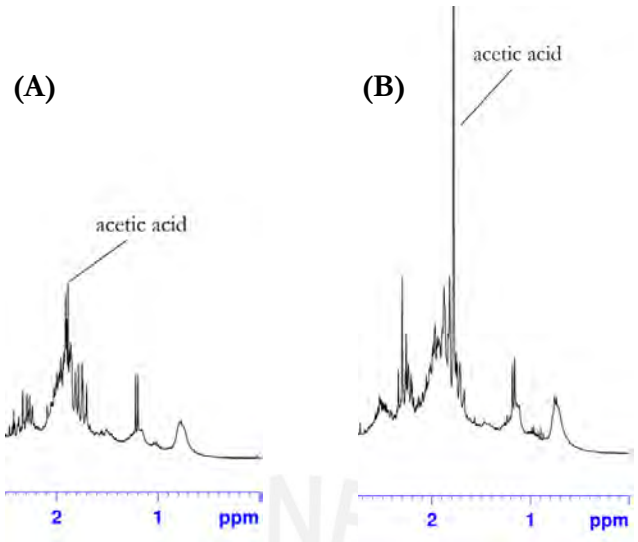


Figure 4.6. Expansion of the ^{1}H-NMR spectrum of a coffee extract from 0 to 3 ppm with (A) and without (B) lyophilization treatment.

Signals in the region from 6 to 10 ppm are better resolved (**Figure 4.7**) and less overlapped than those at higher field (**Figure 4.3**). These signals are well-reported, and were assigned to the aromatic protons from caffeine, trigonelline, 1-methylpyridinium and nicotinic acid, the olefinic protons of caffeoylquinic acids (CQAs), and the non-carboxylic proton of formic acid. The multiplets at 8.1 – 8.3 ppm and 7.8 – 7.9 ppm and the singlet at 6.5 ppm could not be identified.

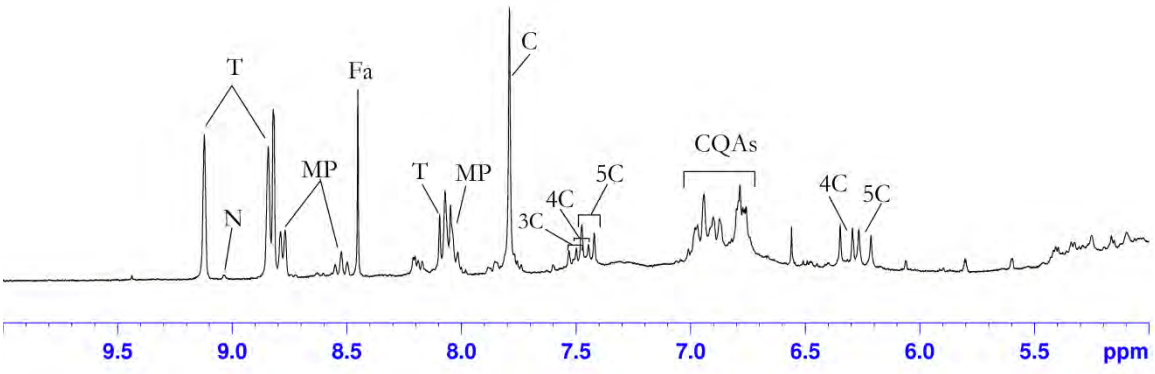


Figure 4.7. Expanded ^{1}H-NMR spectrum expansion from 5 to 10 ppm of a representative coffee extract under study. Compounds identified: T (trigonelline), N (nicotinic acid), MP (1-methylpyridinium), Fa (formic acid), C (caffeine), CQA (caffeoylquinic acid), 4C (4-caffeoylquinic acid), 5C (5-caffeoylquinic acid).

Trigonelline in the coffee extract shows three signals in this region instead of four, as in the individual standard (**Table 4.3**), a singlet at 9.12 ppm (H-2), a doublet (which integrates for two

protons with respect to H-2) at 8.82 ppm corresponding to the aromatic protons H-4 and H-6, and a triplet at 8.09 ppm corresponding to H-5. All of these signals correlate to each other, and to the singlet at 4.44 ppm in the COSY spectrum (**Figure 4.4**). The doublet at 8.77 ppm, which corresponds to the protons H-2 and H-6 of 1-methylpyridinium, overlaps with a signal associated with trigonelline. This doublet correlates to a triplet at 8.04 ppm which corresponds to H-3 and H-5 in 1-methylpyridinium. The latter also overlaps with a signal from trigonelline. The triplet at 8.52 ppm corresponds to the H-4 proton, and, as expected, correlates in the COSY spectrum with the triplet at 8.04 ppm. The only aromatic proton of caffeine is seen as a singlet at 7.82 ppm (H-8), correlating with the N7-methyl group at 3.86 ppm. Singlets at 9.04 (H-2) and 8.45 (H-1) ppm confirm the presence of formic acid and nicotinic acid, respectively. Those assignments were corroborated through a careful spiking experiment. As shown in **Figure 4.8**, the formic acid signal was also diminished after lyophilization.

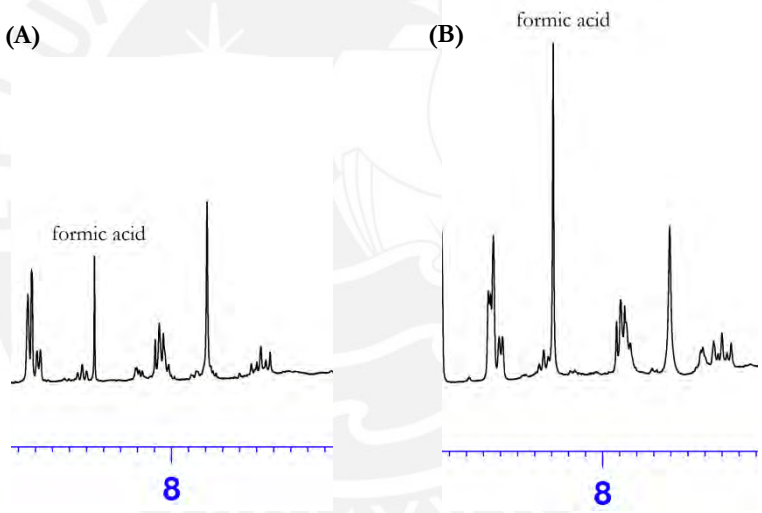


Figure 4.8. Expanded region of the ^{1}H-NMR spectra (7 - 9 ppm) of the coffee extract sample with (A) and without (B) lyophilization treatment (the spectra are on the same scale).

Given that only the isomer 5-CQA (5C) is available commercially, the assignment of the other chlorogenic acid isomers, 4-CQA (4C) and 3-CQA (3C), was made by studying the detailed *J* value and chemical displacement analysis reported by Wei *et al.* (2010) for green coffee and considering their relative abundance (see **Table 1.4**). For 5-CQA it was possible to assign the two doublets at 6.25 ppm and 7.47 ppm as corresponding to the olefinic protons H-1 and H-2, and an additional doublet at 6.92 ppm corresponding to the aromatic proton H-5 (**Table 4.4**). The distinction between H-1 and H-2 was possible from the analysis of the HSQC spectrum (**Figure 4.9**). From this data it is clear that H-1 correlates with a carbon signal at 115 pm, whilst H-2 correlates with at 146 ppm due to the influence of the aromatic group.

The ^{13}C-NMR assignments of the carbons directly bonded to the protons discussed above were also made from the HSQC spectral analysis (**Figure 4.9**).

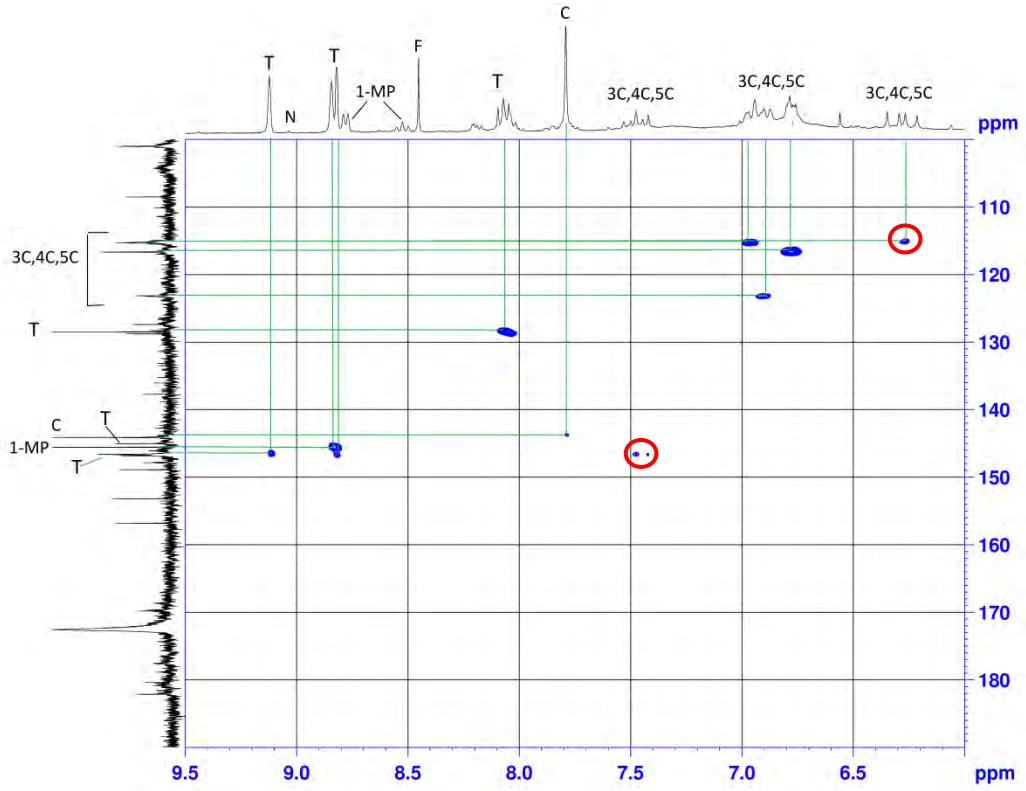


Figure 4.9. HSQC spectrum expansion from 6 to 9.5 ppm of a representative roasted coffee bean extract.

In summary, from the above analysis, it is apparent that there are non-overlapped signals in the region 6 – 10 ppm of the ^{1}H-NMR spectrum which may be useful for quantitation purposes. Even though formic acid is an intense signal and free from overlap, the lyophilization treatment affects its intensity in a non-consistent manner, thereby making it impossible to be used in this study for quantitation purposes in this study.

In **Figure 4.10**, the selected signals for each particular compound of interest are depicted. In the case of 5-CQA, the doublet at 6.25 ppm is somewhat overlapped with other signals, including those of 4-CQA. Being the only signal amenable for quantitation, it will be used in these studies. The results will establish its suitability for quantitative use.

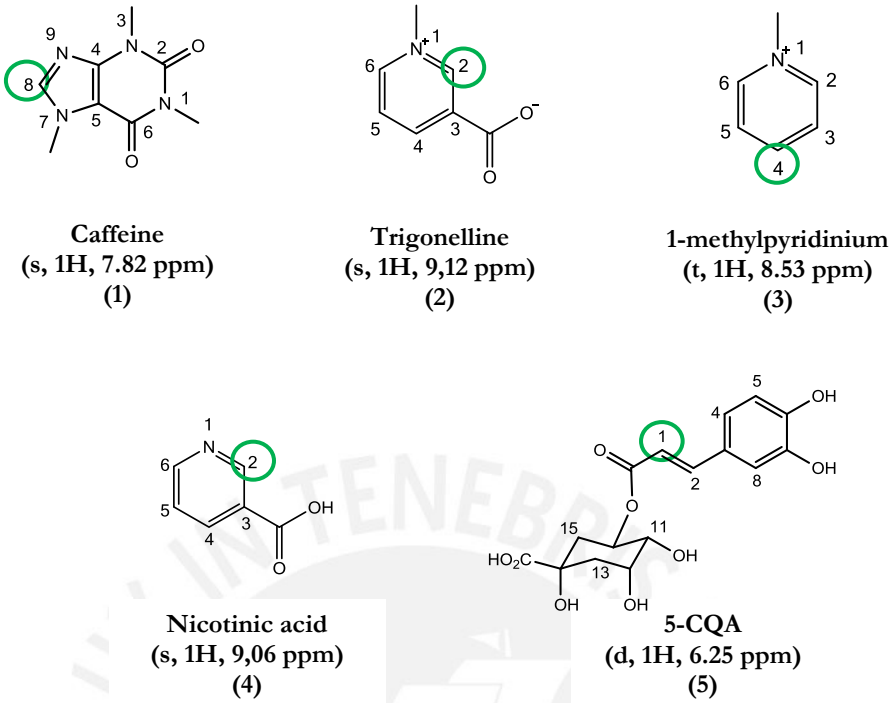


Figure 4.10. Chemical structures of compounds 1 - 5 under study (the protons employed for ^{1}H-NMR quantitation are labeled with circles).

Having selected the NMR signals of interest in the coffee extract spectra, the spectral acquisition parameters need to be optimized.

4.2.3. NMR pulse sequence selection: CPMG vs. NOESY-Presat

Signal broadening, caused by the presence of high molecular weight compounds in the coffee extract, affects the signal resolution of small molecules (Wei *et al.*, 2011). The 1D-CPMG pulse sequence was evaluated for the quantitative study of coffee extracts with the aim to reduce this signal broadening. This technique allowed filtering of the background contributions of the high molecular weight compounds, which display broader resonances than the smaller molecules due to their shorter transverse relaxation times (T_2) (Claridge, 2009). Through this technique, the signals of the fast diffusing species, i.e. the larger compounds, are attenuated or removed, whilst the resonance of the smaller molecules are less affected. In **Figure 4.11,** the 1D-CPMG spectrum of a representative coffee extract is compared to that of the ^{1}H-NMR spectrum using the default parameters.

Through 1D-CPMG analysis, the signals are better resolved: they are sharper and therefore more intense (better S/N). However, a closer inspection of the region from 5 to 10 ppm revealed that the caffeine and the chlorogenic acids signals are severely compromised. This could be explained if the T_2 values of these molecules were similar to those of the high molecular weight molecules (Claridge, 2009). Therefore, the 1D-CPMG acquisition protocol, at the default values used, is not a suitable tool for quantitation.

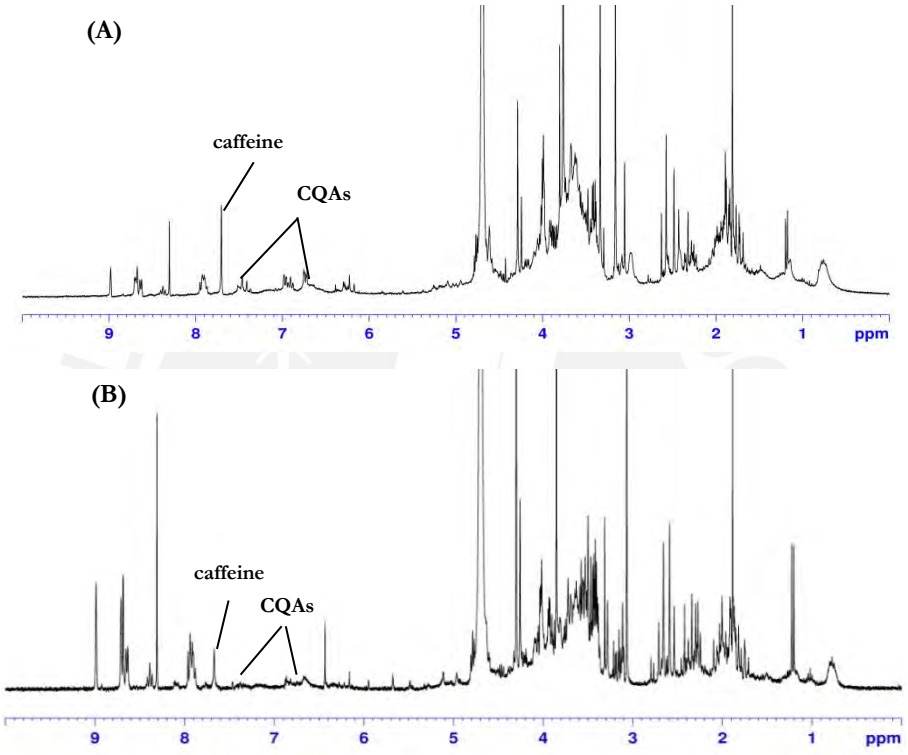


Figure 4.11. Original zg spectra (A) and 1D-CPMG spectra (B) of a representative roasted coffee bean extract.

The NOESY-Presat sequence was employed to minimize the broadening due to the water signal. The pulse sequence involved the irradiation of the frequency associated to the nucleus of interest (H-O-D, H_2O) prior to the excitation and acquisition steps (Claridge, 2009). By suppressing the solvent peak signal, the baseline and the overall resolution of the ^{1}H-NMR spectrum are improved. The signals adjacent to the solvent frequency are inevitably affected (**Figure 4.12**), but none of the signals of interest in this study (the lower field region) pertain to that area of the spectrum, so its potential for quantitative purposes is not diminished by this experimental NMR manipulation.

It is known that pre-saturation sequences such as NOESY-Presat, also alters exchangeable proton signals. However, the sample preparation undertaken (pH control by the use of oxalate buffer and full deuterium exchange by previous treatment with D_2O followed by lyophilization) may diminish this attenuation. All of the NMR data acquisition, including that used for quantitative NMR purposes, was performed with this NOESY-Presat sequence.

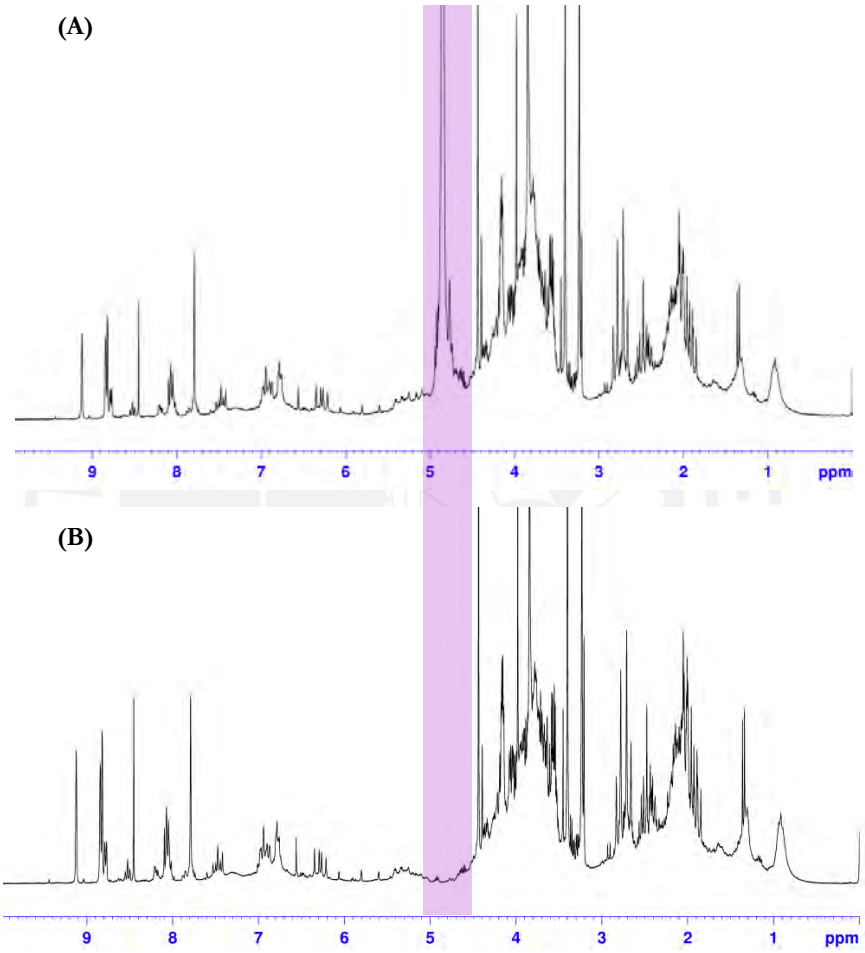


Figure 4.12. Original zg spectrum (A) vs. the NOESY-Presat spectrum (B) of a representative roasted coffee bean extract.

4.2.4. Optimization of acquisition parameters

The ^{1}H-NMR parameters were optimized to assure maximum relaxation of the proton nuclei under study after each data acquisition and to maximize resolution.

The inversion recovery NMR pulse sequence was employed to determine the longitudinal relaxation time (T_1) for the proton signals selected. The T_1 values were calculated in the two types of sample environments: a mixture of standard compounds and the aqueous coffee extract. **Table 4.5** shows the T_1 values obtained.

Table 4.5. Longitudinal relaxation times (T_1) for compounds 1 – 5 using a pulse width of 9 μs.

Compound	δ[a] (ppm)	T_1 (s, coffee sample)	T_1 (s, standard mixture)
Trigonelline	**9.124**	**1.000**	**5.623**
Nicotinic acid	**9.061**	**n.d[b]**	**8.541**
1-Methylpiridinium	**8.588**	**1.979**	**6.401**
Caffeine	**7.821**	**1.183**	**6.080**
5-Caffeoylquinic acid	**6.252**	**1.189**	**0.728**
TSP	**0.000**	**1.456**	**2.793**

[a] Chemical shift of the signal used for quantitation
[b] n.d = not determined due to the very low intensity of this signal in the coffee extract

As can be seen, there is a substantial difference between the T_1 values in these two types of samples. One possible explanation for this behavior could be related to the presence of paramagnetic metal ions in these complex coffee extracts that might be acting as relaxing agents. This statement is supported by literature reports that have demonstrated, through elemental analysis studies (ICP), that the roasted coffee bean samples contain metal ions such as copper(II) (1 - 20 ppm) and iron(III) (2 - 30 ppm) (Anderson and Smith, 2002; Ali-Mohamed and Khamis, 2004), both metals are well-known as relaxing agents in NMR spectroscopy (Claridge, 2009).

Theoretically, it is recommended for NMR-quantitative purposes that the relaxation delay (D_1) be at least five times the longest T_1 value found in the sample under study. This guarantees 99.3 % recovery of longitudinal magnetization (Claridge, 2009). The longest T_1 value attained in the study, **Table 4.5**, is that of the 1-methylpyridinium ion, 1.979 s, therefore, a D_1 value of 12.5 s was set for all coffee samples. However, considering that T_1 of the nicotinic acid standard is the highest, it is quite possible that its T_1 value in the coffee extract may be higher than the one used to set D_1, hence, affecting the NMR quantitation of nicotinic acid.

In the case of the calibration curves employing the mixtures of standards, D_1 was also set to 12.5 s. This decision was based on the fact that (peak area$_{std}$ / peak area$_{TSP}$) using D_1 = 12.5 s and that using D_1 = 40.0 s ($5T_1 = 5 \times 8.541$ s) remain constant in all cases, see **Table 4.6**.

Table 4.6. Absolute areas of the signals from the standard compounds in a mixture with two different D_1 values.

Compound	δ[a] (ppm)	Integral (D_1 = 12.5 s)	Integral (D_1 = 40 s)	Percentage difference (%)
Trigonelline	9.12	15439183	15792931	2.29
Nicotinic Acid	9.06	935995	941259	0.56
1-Methylpyridinium	8.53	5984830	5909807	1.25
Caffeine	7.79	24494732	25437001	3.85
5-Caffeoylquinic acid	6.25	3678879	3878425	5.42

[a] Chemical shift of the signal used for quantitation

A number of 32 scans was found to be sufficient to obtain an adequate S/N ratio in the coffee extract sample. The receiver gain (RG) was set to 90.5 with the aim to obtain sufficiently intense signals to overcome digitization noise and to improve the S/N ratio, without increasing sample concentration (Claridge, 2009). All other parameters, such as the number of data points, line broadening and spectral width were set as default, as mentioned in the experimental section. The values selected for all the parameters allowed for reduction in the spectra recording time without compromising resolution. The time involved in the acquisition of each spectrum was 11 min.

Pulse width optimization for the 90° pulses was performed for the signals of 1-methylpyridinium, caffeine and trigonelline in order to evaluate its influence in the quantitative aspects of the NMR experiment. According to the results shown in **Table 4.7,** all the signals differ in almost 1 μs from the default value (9 μs). Additionally, it can be seen that pulse width is a sample-dependent parameter, but it does not change among the different signals, which indicates that, if necessary, pulse width optimization can be performed only for one signal.

Table 4.7. 90° pulse width optimization for each signal employed for quantitation in the different coffee extracts.

Coffee extract	Pulse width 1-methylpyridinium (μs)	Pulse width Caffeine (μs)	Pulse width Trigonelline (μs)
Instant coffee	10.00	10.00	10.00
Hayabamba coffee a	10.13	10.13	10.13
Hayabamba coffee b	10.13	10.13	10.13
APU Espresso	10.00	10.00	10.00
APU Gourmet	10.00	10.00	10.00
APU Classic	9.88	9.88	9.88
Standard mixture	9.65	9.65	9.65

4.2.5. Quantitation of selected compounds in various coffee samples

The ^{1}H-NMR spectra of the six coffee extracts under study are shown in **Figure 4.13**. In general, the spectra show a similar profile, and all signals previously assigned by 2D NMR are easily recognized.

However, on a closer inspection, an important difference between the specialty coffees and the instant coffee (IC) is depicted: the presence of a singlet at 9.44 ppm, two doublets at 7.52 and 6.68 ppm and a singlet at 5.42 ppm (red arrows in **Figure 4.14**) which matches with the ^{1}H-NMR signals reported by Charlton *et al*. (2002) in an NMR study of different instant coffees, suggesting that these signals might correspond to the aldehydic proton, the furan protons H-3 and H-4 and the H-1 methylene proton of 5-hydroxymethylfurfural (5-HMF), respectively (**6** in **Figure 4.13**). The presence of 5-HMF, as discussed later, was confirmed in this study by LC-MS-MS through observation of the ions at *m/z* = 127 and 109.

The compound 5-HMF has been described by others as a quality marker of deterioration in various foods (Perez-Locas and Yaylayan, 2008; del Campo *et al.*, 2010), and hence, its detection and quantitation has recently been highlighted as an important issue in quality control programs. Moreover, 5-HMF is known to promote hepatocarcinogenic activity in mice (Monien *et al*., 2012). Considering the levels reported in instant coffee (0.62 - 6.18 mg / g), and knowing that this compound also exists in other dairy products (honey, vinegars, jam, juices), monitoring 5-HMF in various coffee samples may become important when health issues are taken into consideration. Therefore, the quantitation of 5-HMF was embraced in this study.

Much more variation between the ^{1}H-NMR profiles of the coffee samples was observed at the higher field regions, from 0 to 5 ppm (**Figure 4.14**), in particular, the interval from 3.5 to 4.3 ppm, which is associated with caffeine, quinic acid and polysaccharides. As explained previously, due to the complexity of this region, in the present study, none of the compounds of interest are addressed in that segment of the spectrum.

Two different quantitative NMR methodologies were used to quantify compounds **1** - **5** (**Figure 4.10**), in the coffee samples: the use of standard calibration curves and ERETIC2-based quantitation. Compound **6** was quantified only by ERETIC2. A description of the results attained follows.

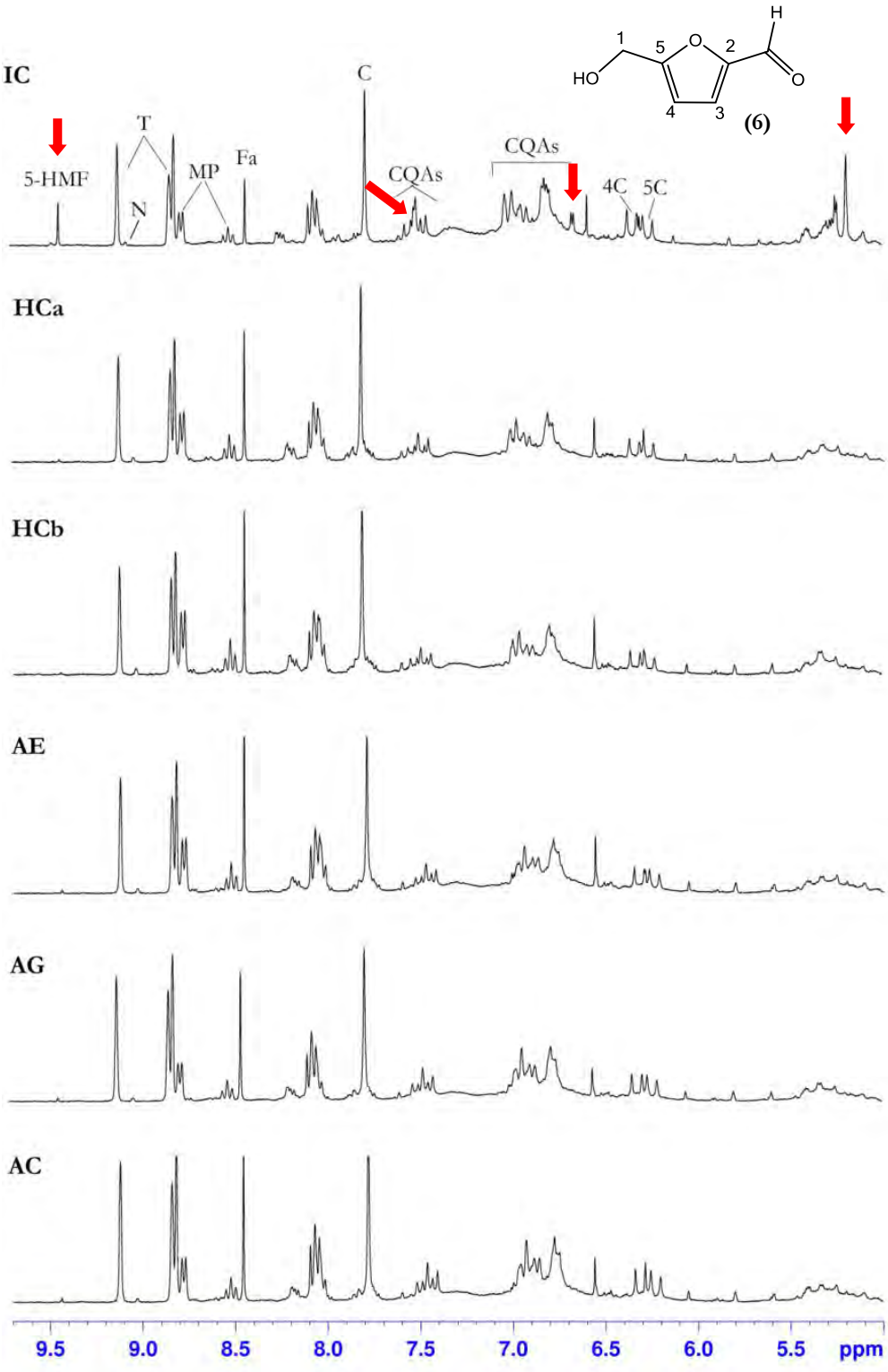


Figure 4.13. Expanded ^{1}H-NMR spectra, from 5 to 10 ppm, of representative samples from each type of coffee studied: IC (Instant coffee), HCa (Huayabamba coffee a), HCb (Huayabamba coffee b), AE (APU Espresso coffee), AG (APU Gourmet coffee) and AC (APU Classic coffee). 5-HMF signals are highlighted with red arrows.

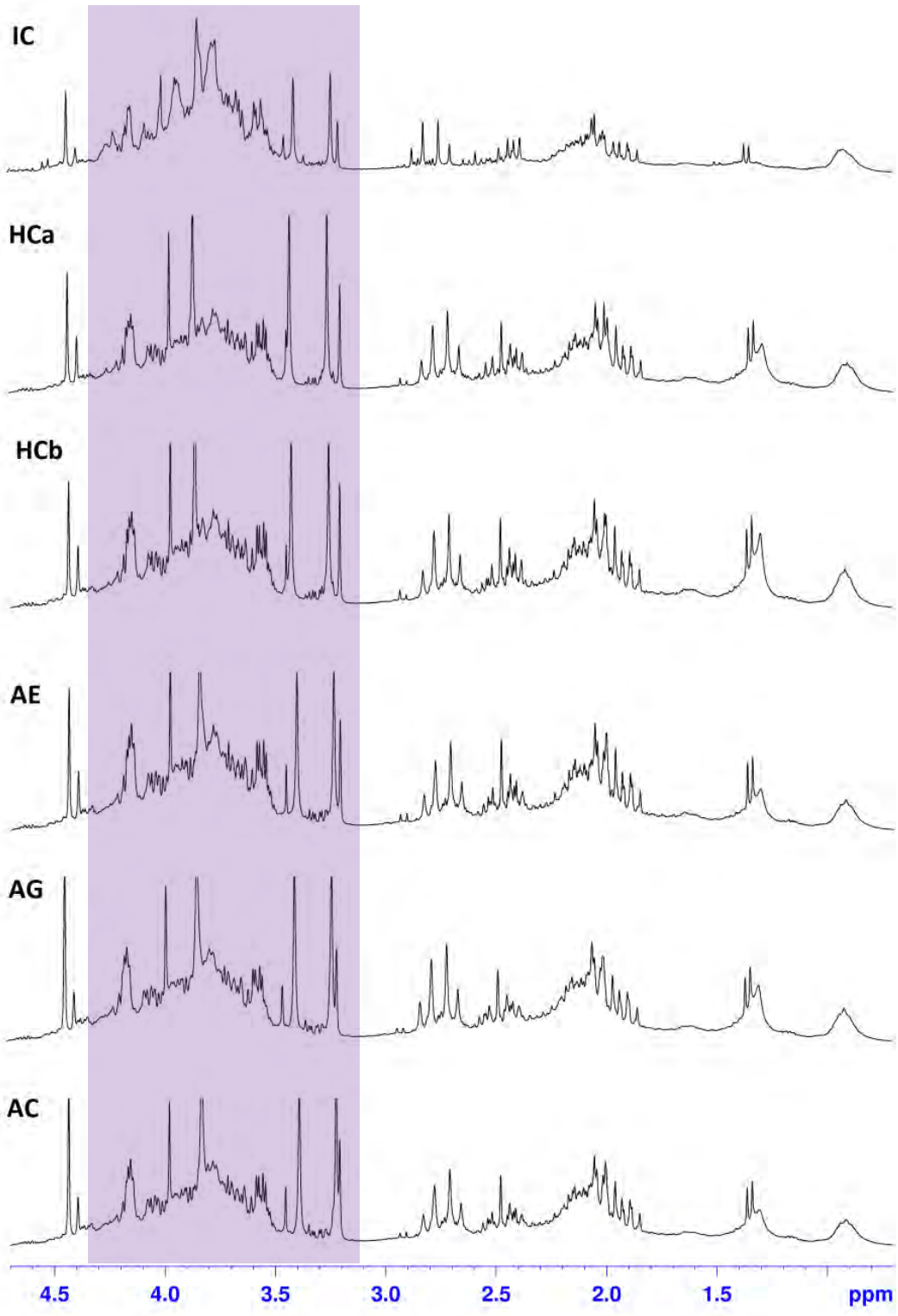


Figure 4.14. Expanded ^{1}H-NMR spectra, from 0 to 5 ppm, of representative samples from each type of coffee studied: IC (Instant coffee), HCa (Huayabamba coffee a), HCb (Huayabamba coffee b), AE (APU Espresso coffee), AG (APU Gourmet coffee) and AC (APU Classic coffee).

Standard Calibration Curves

A mixture of the five compounds at various concentrations, following the preparation protocol (**3.2.2.3**) yielded spectra that, when compared to spectra from the coffee extracts, lead to some important observations.

Although oxalate buffer was used to minimize the pH variation among the samples, the aromatic chemical shifts of 5-CQA were severely shifted among the standard mixtures, as can be appreciated in **Figure 4.15**. This behavior may be the result of the inevitable concentration-dependent interactions between caffeine and chlorogenic acids to form caffeine-chlorogenate complexes in aqueous coffee solutions, leading to slight chemical shift displacements (D'Amelio *et al*., 2009; Wei *et al*., 2012).

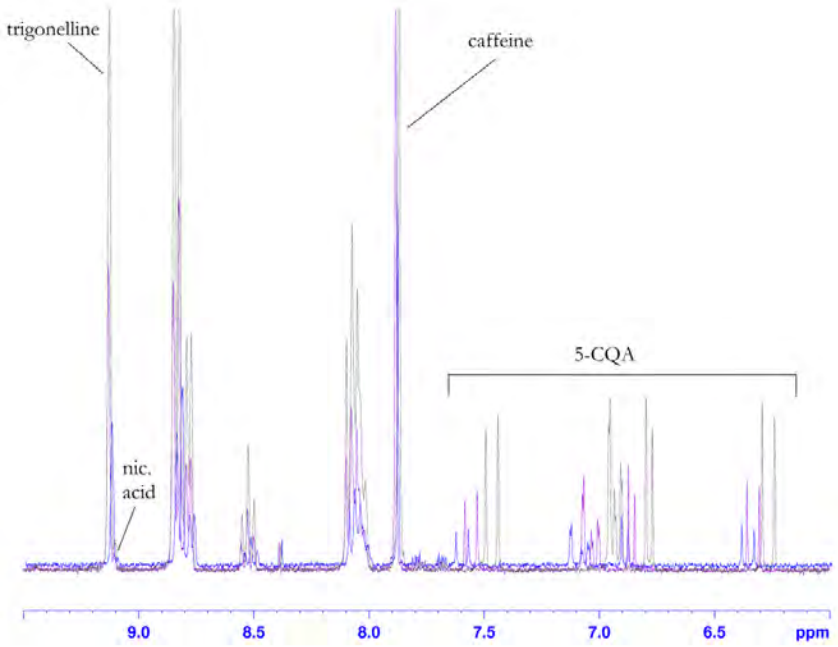


Figure 4.15. ^{1}H-NMR spectra of standard mixtures, at three different concentrations, employed for the standard calibration curves. Concentration-dependent chemical shift variation of chlorogenic acids signals can be observed.

It is important to highlight also that the nicotinic acid standard overlaps in the mixture of standards with trigonelline. This rendered nicotinic acid unfit to be evaluated by NMR calibration curve methodology, and will also affect the trigonelline signal area for quantitation.

The concentration range of all the compounds used to prepare the calibration curves (**Table 4.8**) were assigned taking into consideration the quantitation results obtained by HPLC (**section 4.3**). The linear regression parameters for all four calibration curves indicate good linearity in the concentration range established for each of the four standards used (**Table 4.8**).

Table 4.8. ^{1}H-NMR calibration curves of standard compounds.

Compound	δ[a] (ppm)	Concentration range (mg/mL)	Linear regression	
			y=mx+b	R^2
Trigonelline	9.12	0.237 - 2.001	y = 87.730E6x - 8.859E6	0.9999
1-Methylpyridinium	8.53	0.119 - 1.011	y = 80.539E6x - 4.262E6	0.9977
Caffeine	7.82	0.417 - 3.529	y = 89.605E6x - 31.176E6	0.9994
5-Caffeoylquinic acid	6.27	0.144 - 1.220	y = 72.368E6x + 9.203E6	0.9998

[a] Chemical shift of the signal used for quantitation; R^2 = Correlation coefficient; the results are an average of two independent experiments (n = 2).

The calibration curves were validated using a mixture of standards of a known concentration. Results are shown in **Table 4.9**. The percentage errors attained vary from 1 to 3 %, confirming its accuracy for quantitation.

Table 4.9. Standard calibration curve validation results for standard compounds.

Compound	δ[a] (ppm)	Real concentration (mg/mL)	Calculated concentration (mg/mL)	Percentage error (%)
Trigonelline	9.12	0.6640	0.6804	2.47
1-Methylpyridinium	8.53	0.3374	0.3271	3.05
Caffeine	7.82	1.1542	1.1713	1.48
5-Caffeoylquinic acid	6.25	0.4018	0.4104	2.14

[a] Chemical shift of the signal used for quantitation; the results are the average of two independent experiments (n=2)

When comparing the ^{1}H-NMR spectrum of a mixture of the standard compounds **1** – **5**, shown in red in **Figure 4.16**, against the ^{1}H-NMR spectrum of the coffee extract (blue contour in **Figure 4.16**), an important chemical shift variation, between the signals of the standards in the mixture and those in the real samples is observed. This is particularly relevant in the case of nicotinic acid (N), which separates from trigonelline (T) in the coffee extracts, compared with signal overlap with this compound, in the standard mixture. This variation may be also related to complex

formation in the case of caffeine and chlorogenic acid variation or other concentration-dependent interactions that were not identified in the case of nicotinic acid.

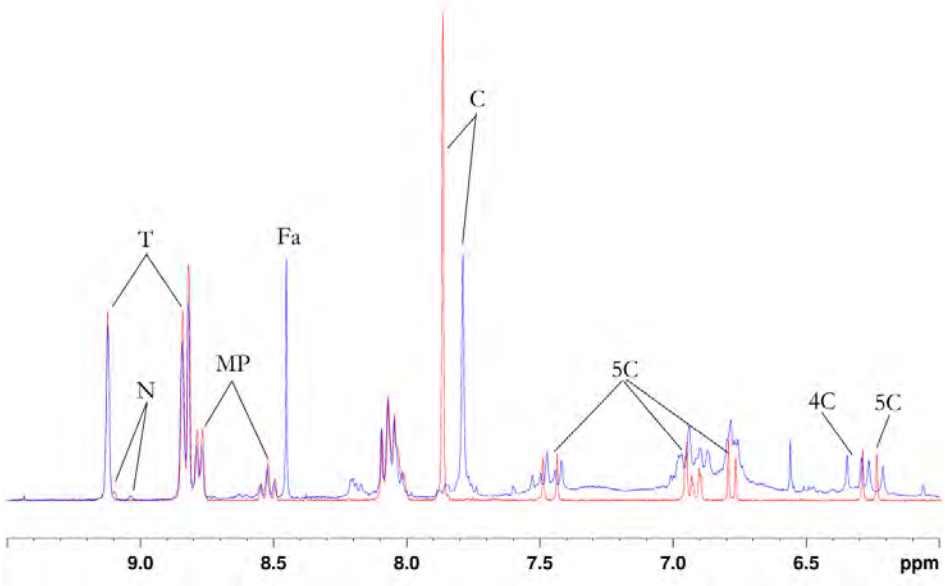


Figure 4.16. ^{1}H-NMR spectrum expansion from 6 to 10 ppm of a representative roasted coffee bean extract overlaid with that of the standard mixture.

Another important variation that can be appreciated in **Figure 4.16** is that the 5-CQA (5C) signal at 6.25 ppm is not sufficiently separated from the 4-CQA isomer in the coffee sample (blue contour). These factors may introduce error in 5-CQA quantitation by NMR.

In summary, trigonelline, 1-methylpyridinium ion, caffeine and 5-CQA, present in the six coffee extracts studied, were quantified using the standard calibration curves described in **Table 4.8**. Baseline correction using an algorithm available in ACD / NMR Processor 12.01 was necessary in order to improve the accuracy of the method; particularly in the case of 1-methylpyridinium (see **Annexes 1 – 12**).The results obtained are shown in **Table 4.10.** The discussion of these data will be addressed after the ERETIC2 methodology, which was also used to quantify these standards in the coffee extracts, is described and discussed.

ERETIC2 methodology

In order to evaluate the applicability of the ERETIC2 tool, available in the Topspin NMR software, for quantitation purposes, this method was used to quantify the selected compounds **1** -

6 in the coffee extracts. Quinic acid (**Figure 4.17**) was chosen as the calibration reference because it fulfills the requirements established for an ERETIC2 reference: it has, as a standard, a longitudinal relaxation time, similar to the T_1 values of the compounds that will be quantified in the coffee extract samples under study (T_1 = 1.89 s), see **Table 4.5**.

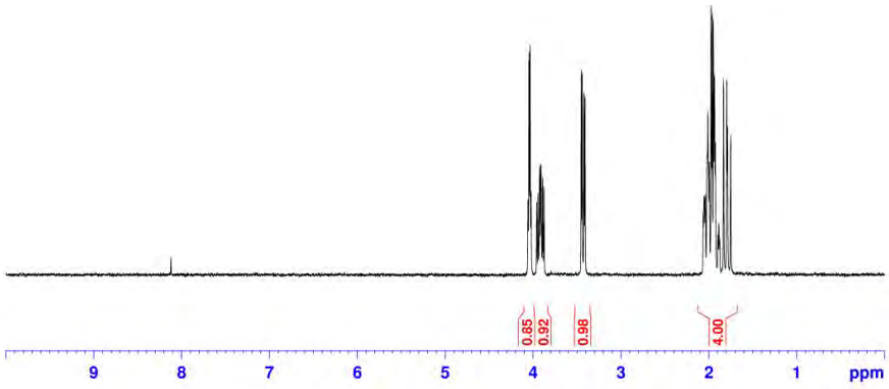


Figure 4.17. ^{1}H-NMR spectra of quinic acid used as a calibration reference for ERECTIC2 quantitation.

ERETIC2, based on PULCON (pulse length based concentration determination), uses the formula described below to calculate the concentration of an unknown analyte (C_{unk}) from the integrated areas of the reference signals (A_{ref}) of a known concentration (C_{ref}) against the areas of the analyte of interest (A_{unk}). This is possible if the 90° pulse has been well-calibrated for all samples. In the formula below, θ_{90}^{unk} and θ_{90}^{ref} represent the pulse needed to obtain a tip angle of 90° for each particular sample, whereas "T" is associated with temperatures and "n" represents the number of scans. The correction factor (k) takes care of incomplete relaxation or differences in receiver gains between experiments.

$$C_{unk} = kC_{ref}\frac{A_{unk}T_{unk}\theta_{90}^{unk}n_{ref}}{A_{ref}T_{ref}\theta_{90}^{ref}n_{unk}}$$

The amounts obtained for compounds **1** – **6** in the six different coffee extracts, using these two different NMR-quantitative approaches, are shown in **Table 4.10**.

In general, the Percentage Differences (PD%) between these two methodologies are within reasonable values, PD% < 15%. In some cases, it varied widely with the sample and the

compound analyzed. No pattern was observed for these variations. That is, the PD% for compounds in the same sample (Huayabamba coffee a, for example) would vary from 8 to 32 %, whereas in instant coffee, it remained almost constant, 8 – 11%. With compounds like trigonelline, the PD% in all coffee samples varied from 3 to 15 %, whereas for 5-CQA, particularly in the case of APU Espresso and Huayabamba coffee, the PD% reached values as high a 33 %.

The highest PD% values are associated with both of the Huayabamba coffees, and with the quantitation of 5-CQA in all samples. The signals of 5-CQA have been previously indicated as troublesome, due to the shifts they experience through complex formations, and to the inevitable overlap with 4-CQA signals. The existence of 5-CQA adducts will unequivocally be highly dependent on the coffee extract matrix.

Even though the PD% between these two methods was high in some particular cases, overall, more than 65 % of the PD% data was below 10 %. In addition, it is important to highlight that the values are the result of the analyses of at least four independent samples (n = 4) and good precision (low standard deviation) was obtained for all the compounds in all samples analyzed.
The experimental ease of using the ERETIC2 methodology instead of NMR-standard calibration curves, justified its use to address the 5-HMF quantitation in the coffee samples (**Table 4.10**), especially considering that a 5-HMF standard was not readily available in the laboratory.

The singlet at 9.44 ppm, corresponding to the aldehydic proton H-1 of 5-HMF (see structure in **Figure 4.13**), was used for quantitation. The result attained for instant coffee (1.959 ± 0.075 mg / g of dry aqueous extract) was comparable to the data reported by del Campo *et al.* (del Campo *et al*., 2010) in the ^{1}H-NMR analysis of different brands of instant coffee. As can be seen in **Table 4.10**, the content of 5-HMF in specialty coffee extracts is very low in comparison to the amounts found in instant coffee.

The quantitation of the compounds **1** – **6** in all coffee samples studied showed that there is a significant difference among the concentrations of compounds present in the APU coffees, Huayabamba coffees and instant coffee. In the case of trigonelline, APU Gourmet and APU Classic show significantly greater amounts of this compound than the other coffees, which may be an indication of a better quality (Stadler *et al.,* 2002 b; Wei *et al.*, 2011). APU Espresso differentiates from APU classic only in the roasting degree, and as such it shows a 25% concentration decrease in trigonelline. The result is consistent with the well-reported thermal degradation of trigonelline during roasting (Stadler *et al.,* 2002 a,b). However, this change does

not take place in the case of Huayabamba coffees a and b, despite their different roasting times. These results confirm the diverse behavior probably due to changes in the chemical composition among coffees of different regions (Jaen - Cajamarca vs. Huayabamba - Amazonas).

Both nicotinic acid and 1-methylpyridinium contents were higher for coffees with longer roasting times. This increase was particularly significant for nicotinic acid which shows a 25% variation. Results are in accordance with previous studies (Stadler *et al.,* 2002 a), which suggests that the concentration of these compounds increases during roasting as a result of trigonelline thermal decomposition.

APU coffees also show noticeably higher amounts of caffeine than the other coffees examined. This compound is known to contribute, as well as trigonelline, to the bitterness of the coffee brew (almost 30% of the bitter taste is associated with caffeine) (Clarke and Vitzthum, 2001). The variation among the samples was of the order of 10% (Wei *et al.*, 2012), a value which coincides with similar reports available for *C. arabica* with different roasting degrees (Casal *et al*., 2000 a).

Finally, the 5-caffeoylquinic acid content varies significantly among all of the samples. The greater contents were found for the APU coffees Gourmet and Classic, whilst Huayabamba shows the lower amounts. A consistent trend in 5-caffeoylquinic acid variation with the roasting time was not observed for comparable samples. However, in the case of APU coffee there is a concentration decrease of ~15 %, a value that can be corroborated with the reported data, which indicate that the concentration of 5-caffeoyl quinic acid decreases with increasing roasting times (Blumberg *et al.*, 2010). The same can not be said for Huayabamba coffees.

In the next section, a validated HPLC-DAD methodology is used to evaluate the NMR results presented here.

4.1. Quantitative determination by HPLC

High performance liquid chromatography (HPLC) is the most widely applied technique for the quantitation of small, non-volatile molecules such as compounds **1** – **5**. Several strategies exist for this purpose, including some protocols already available for coffee analysis (Casal *et al*., 2000 a,b; Ky *et al*., 2001; Yen *et al*., 2005; Alves *et al*., 2006). Therefore, this technique was selected to validate the results obtained by the non-classical NMR approach, discussed previously.

Table 4.10. Contents[a] of compounds 1 - 6 quantified by ^{1}H-NMR standard calibration curves as compared with ERETIC2 in six different types of coffee.

Coffee extract		Trigonelline (1)			Nicotinic Acid (2)[b]	1-methylpyridinium (3)		
	Roasting time	NMR StCC	ERETIC2	PD[c]%	ERETIC2	NMR StCC	ERETIC2	PD[c]%
Instant coffee	unknown	10.258 ± 0.235	10.416 ± 0.242	1.54	0.184 ± 0.022	2.186 ± 0.126	2.744 ± 0.100	25.53
Hayabamba coffee a	t1[d]	10.644 ± 1.197	11.475 ± 0.802	7.81	0.289 ± 0.042	3.561 ± 0.318	3.605 ± 0.284	1.24
Hayabamba coffee b	t1[d] + 2min	10.679 ± 0.445	10.734 ± 0.500	0.52	0.426 ± 0.015	4.451 ± 0.147	4.654 ± 0.169	4.56
APU Espresso	t2[d] + 2min	14.016 ± 0.058	14.107 ± 0.486	0.65	0.452 ± 0.017	4.823 ± 0.089	5.018 ± 0.260	4.04
APU Gourmet	t2[d]	18.522 ± 0.174	19.007 ± 0.205	2.62	0.359 ± 0.020	3.965 ± 0.112	4.489 ± 0.086	13.22
APU Classic	t2[d]	19.537 ± 0.225	20.061 ± 0.290	2.68	0.384 ± 0.019	4.459 ± 0.160	4.912 ± 0.055	10.16

Coffee extract	Roasting time	Caffeine (4)			5-caffeoylquinic acid (5)[e]			5-hydroxymetylfurfuraldehyde
		NMR StCC	ERETIC2	PD[c]%	NMR StCC	ERETIC2	PD[c]%	ERETIC2
Instant coffee	unknown	20.822 ± 0.700	22.635 ± 0.819	8.71	16.251 ± 1.002	21.783 ± 0.648	34.04	1.959 ± 0.075
Hayabamba coffee a	t1[d]	25.325 ± 2.168	24.259 ± 1.233	4.21	14.235 ± 1.835	15.147 ± 0.813	6.41	n.d[f]
Hayabamba coffee b	t1[d] + 2min	28.035 ± 1.557	25.863 ± 1.063	7.75	12.690 ± 0.887	16.319 ± 1.206	28.60	n.d[f]
APU Espresso	t2[d] + 2min	31.322 ± 0.393	31.575 ± 0.393	0.81	16.759 ± 0.281	21.698 ± 1.125	29.47	n.d[f]
APU Gourmet	t2[d]	33.877 ± 0.347	35.547 ± 1.633	4.93	22.996 ± 0.643	26.547 ± 1.326	15.44	0.174 ± 0.007
APU Classic	t2[d]	39.614 ± 1.475	39.500 ± 0.755	0.29	25.977 ± 0.593	30.718 ± 0.427	18.25	0.183 ± 0.032

[a] Values are expressed in mg/g of ground material with at least n=4 for ^{1}H-NMR and n=3 for HPLC ± standard deviation; [b] Values may be underestimated due to the low S/N ratio. This was confirmed by the DAD-HPLC results discussed later (Table 4.14); [c] PD = Percentage difference; [d] proprietary information; [e] Values may be overestimated due to the overlap of the signal with other CQA isomers. This was confirmed by the DAD-HPLC results discussed later (Table 4.14); [f] n.d = not detected.

In some of the studies available in the literature, the quantitation of compounds, such as trigonelline, nicotinic, caffeine and chlorogenic acids, using various solvents (**Table 2.1**) allowed validation (Farah *et al*., 2005; Alves *et al*., 2006; Tavares *et al*., 2006; Louarn *et al*., 2001; Fujioka and Shibamoto, 2008). As indicated earlier, when discussing **Table 2.1**, the limitation of most of these protocols is related to the solvents used, which are not amenable for LC-MS analysis, and to the fact that the columns used will not facilitate the selective retention of 1-methylpyridinum ion, in the presence of trigonelline and nicotinic acid. The identification of 1-methylpyridinium by NMR in the samples studied here, suggested the need for a different HPLC column. In the literature, the HPLC-DAD protocol used for the quantitation of this cationic compound in coffee samples required an ion exchange column (Stadler *et al.* 2002 b). The objective of that study was only the detection and quantitation of alkylpyridinium ions. Another procedure is available in the literature which allows for the quantitation of trigonelline in the presence of 1-methylpyridinium ion in urine samples. This method required a hydrophilic liquid interaction chromatography column (HILIC column (Lang *et al*., 2010), column limited to very polar molecules.

In this study, the simultaneous separation of polar and less polar molecules was needed. Hence a different type of column was necessary. Knowing that a regular C-18 column did not resolve cleanly trigonelline and 1-methylpyridinium ion (Maruenda *et al.*, 2012), and keeping in mind that all the compounds of interest contain an aromatic skeleton, the phenyl-hexyl-Luna C18 column represented a good option. This phenyl moiety could be useful to selectively interact, through π-π bonding, with the aromatic compounds being pursued. The solvent system selected for elution contained formic acid 0.3%, pH = 2.4 and methanol, since they facilitate LC-MS analysis. The latter study was used to corroborate the identity of other compounds present in the extract, as well as to confirm that no other compound lies underneath the peaks used for quantitation.

The HPLC-DAD methodology developed as described above permitted the quantitation of all five standards with satisfactory precision, sensitivity and reproducibility results. These results are discussed in the following pages.

4.1.1. HPLC-DAD method validation

Adequate separation among the five standards selected for quantitation was successfully achieved in 30 min, by adapting an HPLC method previously developed by Maruenda *et al*. (2012). The

chromatograms are shown in **Figure 4.18**. 1-Methylpyridinium iodide and nicotinic acid are monitored at 260 nm, trigonelline and caffeine at 270 nm, and 5-CQA at 330 nm. UV spectra of these five compounds are shown in **Figure 4.19**.

The linear regression parameters obtained for the five different calibration curves indicate a good linear correlation in the concentration range established for each compound (**Table 4.11**). Moreover, LOD values in the range of 0.203 - 1.472 µg/mL and LOQ values of 0.676 - 4.907 µg / mL **(Table 4.11)** indicate good sensitivity. Reproducibility, in terms of retention time and peak area, was verified by intra-day and inter-day repetitive analysis (n=5) with satisfactory results, displaying a coefficient of variation (CV %) below 2 %, except for 1-methylpyridinium iodide, which reached 3.2 % (**Table 4.12**).

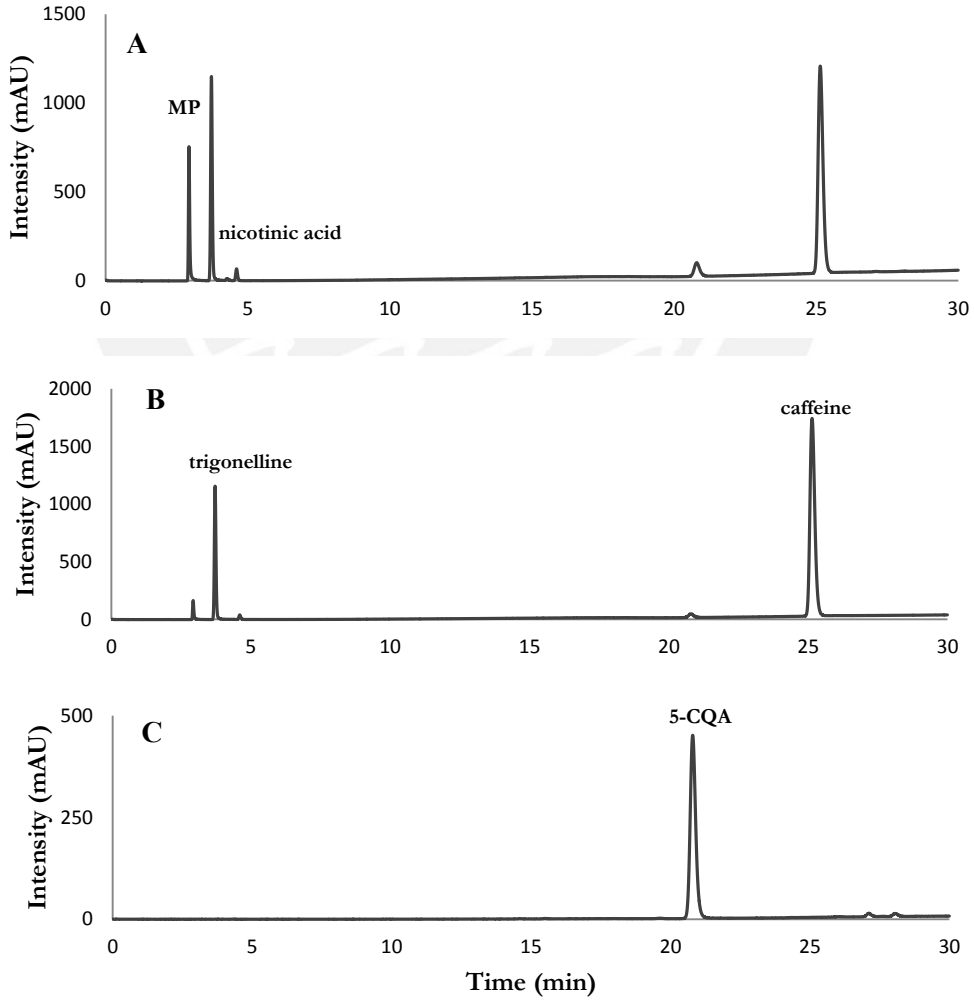


Figure 4.18. Representative HPLC chromatograms at (A) 260 nm, (B) 270 nm and (C) 330 nm of the standard compounds under study. MP = 1-methylpyridinium iodide.

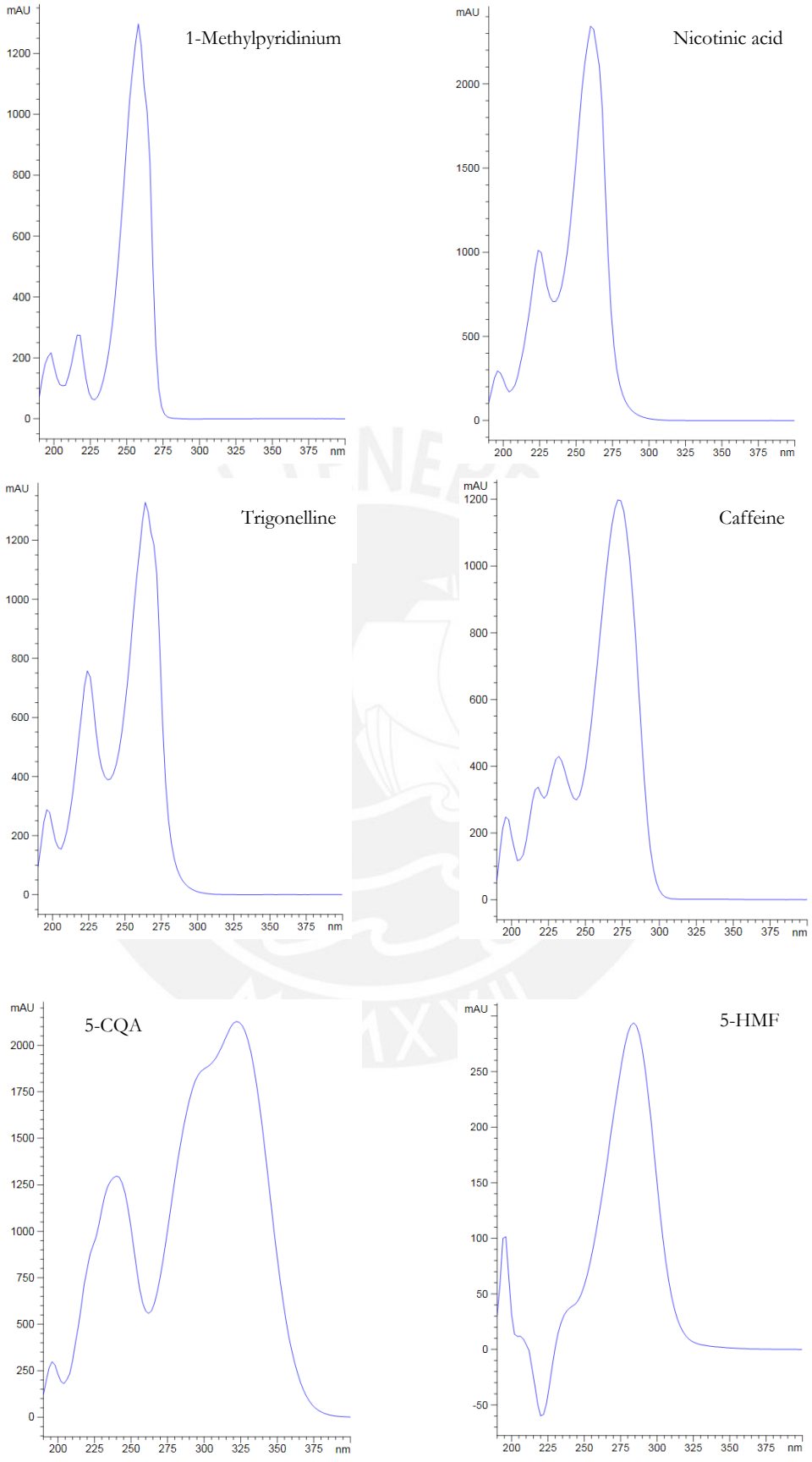


Figure 4.19. UV spectra of compounds 1 – 6.

Table 4.11. Calibration curves of analytes 1 - 5 and the sensitivity of the HPLC-DAD method.

Compound	Concentration range (µg/mL)	Linear regression		LOD (µg/mL)	LOQ (µg/mL)
		y=mx+b	R^2		
Trigonelline	50.3 - 402.4	2082.5x - 14.265	0.9998	0.203	0.676
Nicotinic Acid	1.91 - 15.24	2602.1x - 2.2324	0.9990	0.808	2.694
1-methylpyridinium	27.1 - 216.8	2550.5x - 28.472	0.9996	0.833	2.778
Caffeine	90.27 - 722.2	5657.6x - 33.295	0.9994	1.472	4.907
5-caffeoylquinic acid	30.15 - 241.2	9136.4x + 81.055	0.9965	1.379	4.597

[a] Correlation coefficient; [b] LOD=Limit of detection (S/N=3); [c] LOQ=Limit of quantitation (S/N=10)

Table 4.12. Precision of the retention time and peak area of the analytes 1 – 5[a] in the HPLC-DAD method.

Compound	Retention time (min)	Intra-day precision (CV% n=5)		Inter-day precision (CV% n=5)	
		Retention time	Peak area	Retention time	Peak area
Trigonelline (1)	3.722	0.016	0.786	0.022	1.887
Nicotinic Acid (2)	4.606	0.030	0.597	0.014	0.822
1-methylpyridinium (3)	2.936	0.040	0.577	0.326	3.213
Caffeine (4)	25.111	0.181	0.462	0.174	1.385
5-caffeoylquinic acid (5)	20.853	0.081	1.155	0.131	1.563

[a] Concentrations of the analytes were in the range of 0.062 to 1.860 mM.

The results in terms of sensitivity and reproducibility for caffeine, trigonelline, nicotinic acid and 5-CQA are comparable to another HPLC method reported in the literature for the quantitation of these four compounds in roasted coffee bean extracts (Alves, 2006). In the case of the 1-methylpyridinium ion, a major product of trigonelline decomposition during roasting (Stadler *et al*., 2002 a, b), only a few studies have addressed its quantitation. To the best of our knowledge, none of these studies included a validated methodology for the 1-methylpyridinium cation in the presence of trigonelline, nicotinic acid, caffeine and 5-CQA - except for the study developed by Maruenda *et al.* (2012). Hence, the HPLC method developed in this study represents the first validated HPLC protocol for the simultaneous quantitation of these five particular compounds in coffee.

To evaluate the accuracy of the validated-HPLC standard calibration curves, a sample containing known concentrations of the five standards was prepared and analyzed, obtaining errors of 1 %, except for nicotinic acid, which had an error of 3.2 % (**Table 4.13**).

Table 4.13. Standard calibration curve validation results for compounds 1 - 5.

Compound	Real concentration (μg/mL)	Calculated concentration (μg/mL)	Percentage error (%)
Trigonelline	187.2	186.7	0.28
Nicotinic Acid	7.62	7.37	3.20
1-Methylpyridinium	100.8	102.0	1.24
Caffeine	380.8	380.0	0.21
5-Caffeoylquinic acid	133.3	134.3	0.77

4.1.1. HPLC analyses of coffee samples

4.1.1.1. Qualitative analysis by HPLC-DAD and LC-MS

The HPLC profiles at 270 nm and 330 nm for each coffee extract are displayed in **Figures 4.20** and **4.21** (for more detailed information **see Annexes 13 – 18**). A preliminary inspection of the six chromatograms at 270 nm (**Figure 4.20**) allowed the identification of an additional, and well-resolved, signal of all the coffee samples, between the signal of 1-methylpyridinium ion and that of trigonelline (highlighted in purple in **Figure 4.20**). Additionally, an intense signal at 12 min is present only in the chromatogram of the instant coffee. The UV of this compound (**Figure 4.19**) resembled that of 5-HMF with a maximum molar absorptivity at 280 nm (Lin *et al*., 2012). At 330 nm, **Figure 4.21**, all the samples showed almost the same HPLC - profile. The peaks at 23.3 and 23.7 min could not be resolved by the developed method (highlighted in purple). In order to unequivocally identify the nature of all of these signals, an LC-MS study was conducted.

Figure 4.22 shows the LC-MS TIC spectrum (positive ion mode) profile of instant coffee and **Figure 4.23** the MS and MS/MS spectra for the signal at 12.1 min (peak 7). The ion $[M+H]^+$ at *m/z* 127.4 and its MS/MS fragment at *m/z* 109.5, due to the loss of a water moiety, confirm the presence of 5-HMF (MW = 126 g / mol), previously suggested by NMR (see section **4.2.4**). This conclusion is based on an earlier study performed by Lin *et al.,* (2012), who identified 5-HMF in vinegar through a detailed NMR and LC-MS/MS analysis. Hence, the unknown signal at 12 min in **Figure 4.20** (peak 7, in **Figure 4.22**), corresponds to 5-HMF. As mentioned earlier, 5-HMF has been previously reported in instant coffee by del Campo *et al.* (2010).

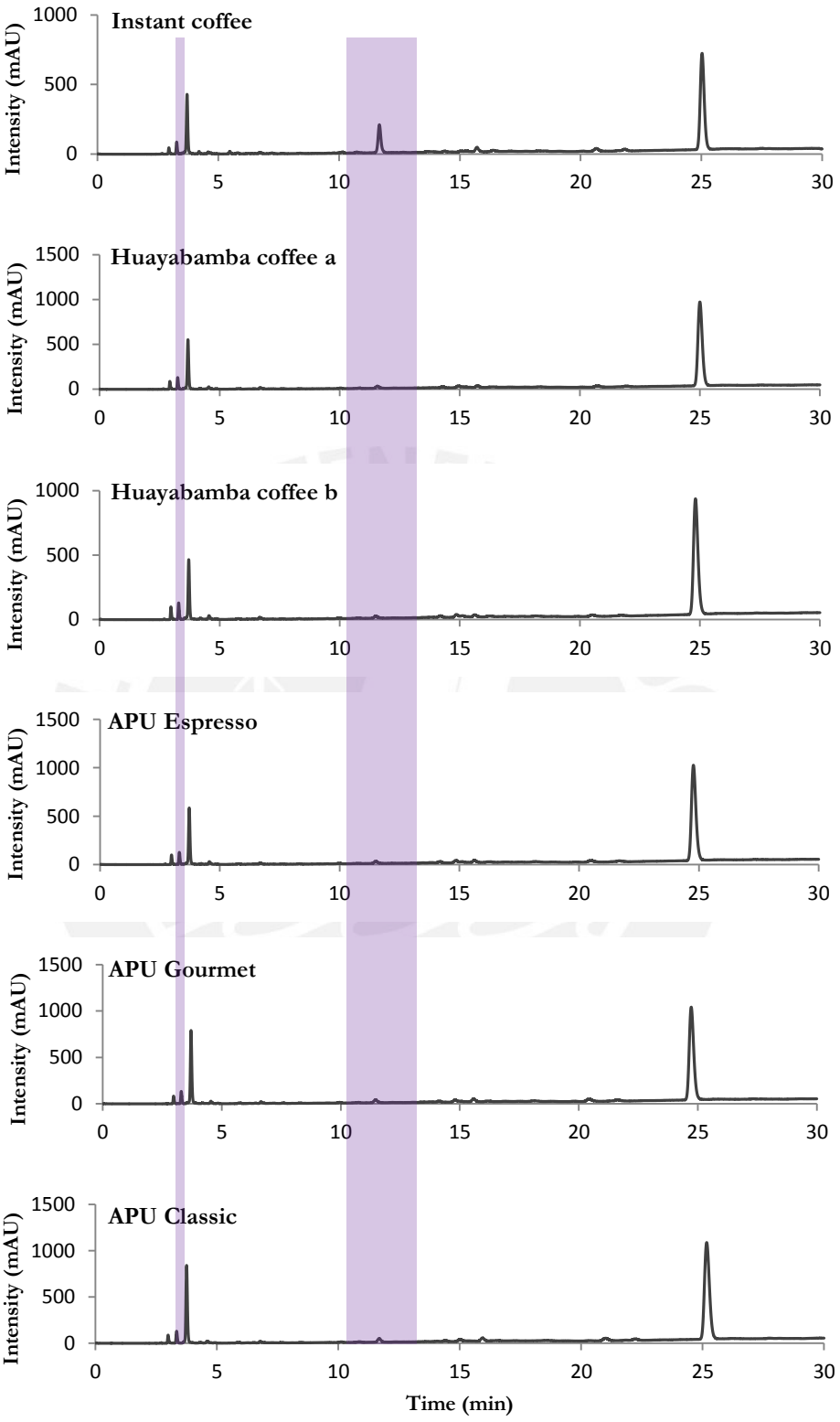


Figure 4.20. Representative HPLC chromatograms at 270 nm of the six coffee extracts under study.

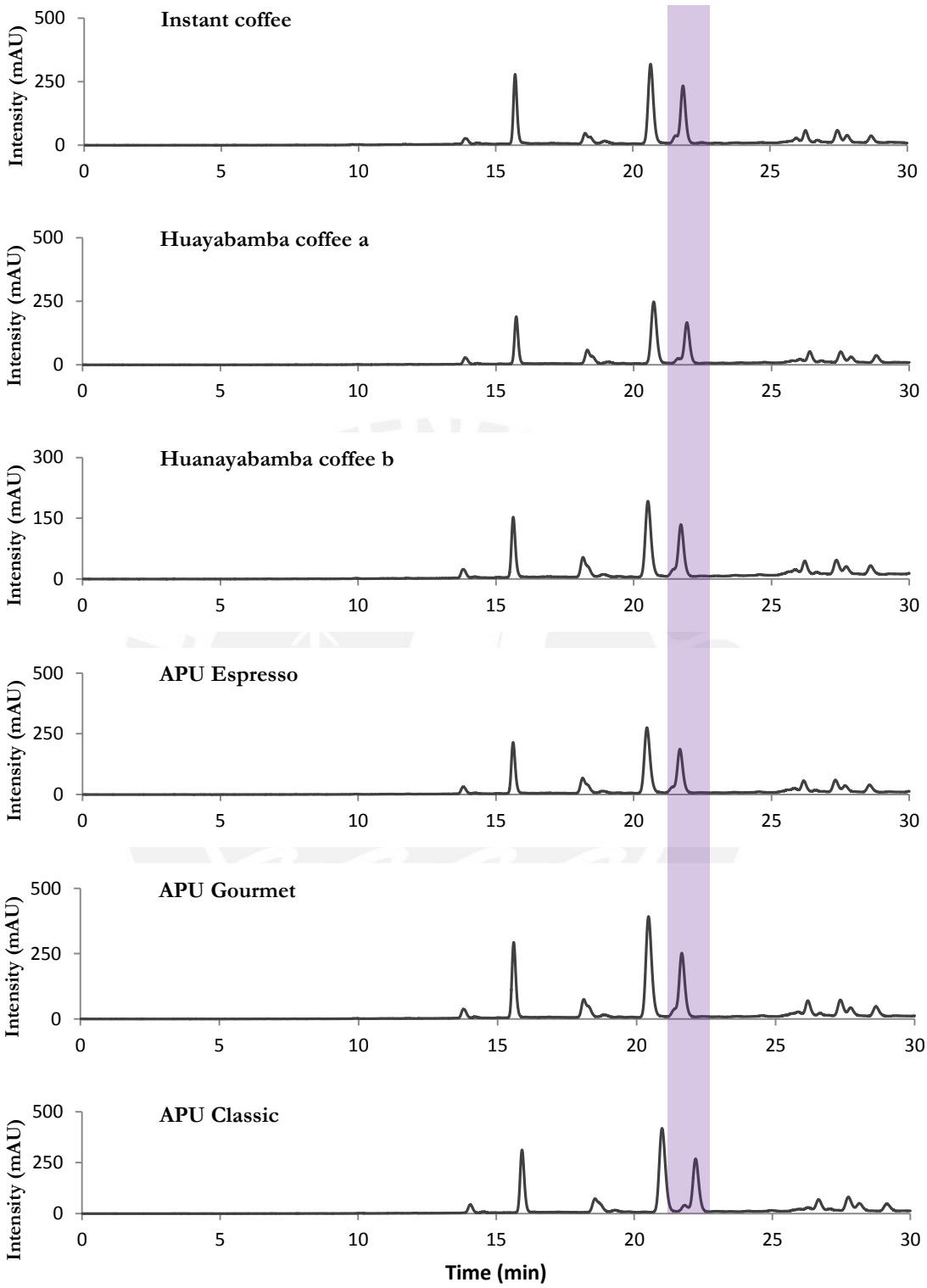


Figure 4.21. Representative HPLC chromatograms at 330 nm of the six coffee extracts under study.

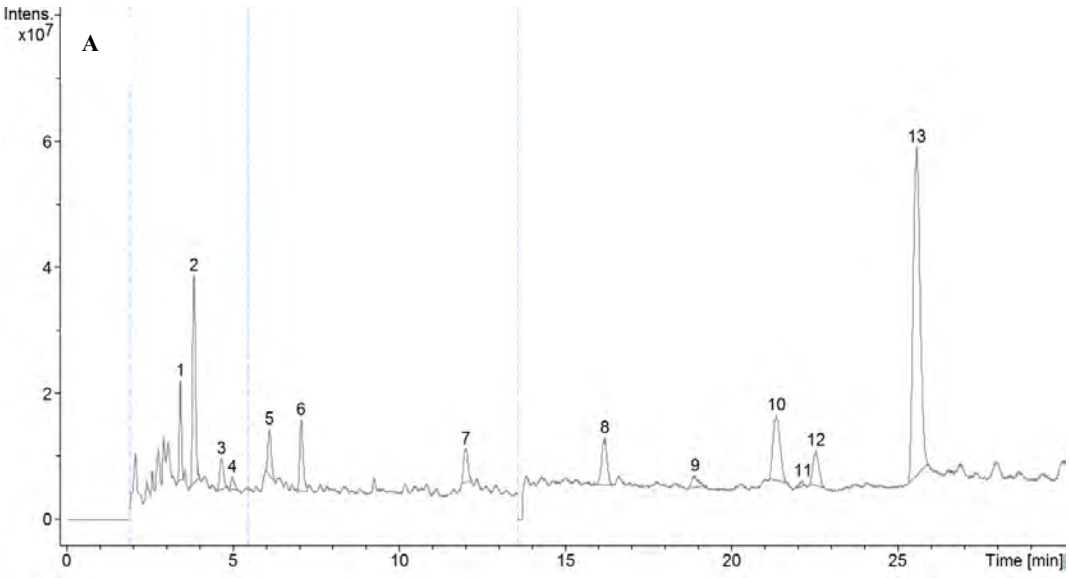


Figure 4.22. LC-MS spectrum of the instant coffee extract: (1) quinic acid, (2) trigonelline, (3) nicotinic acid, (7) 5-HMF, (8) 3-CQA, (10) 5-CQA (11) FQA and (13) caffeine. Peaks 4, 5, 6, 9 and 12 were not identified.

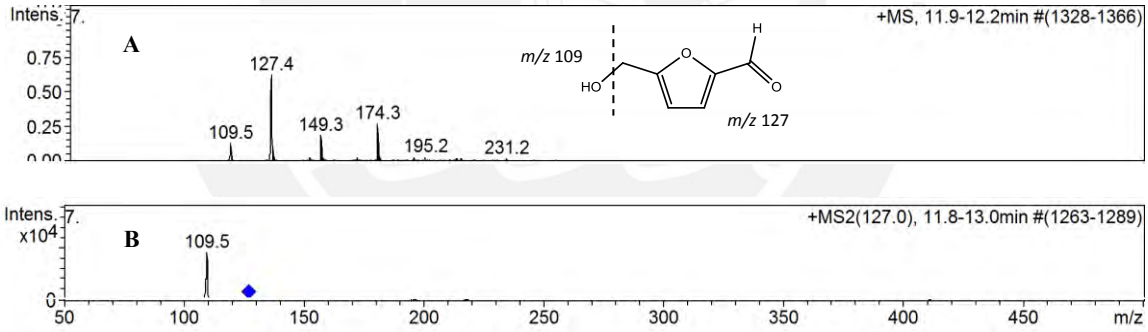


Figure 4.23. MS (A) and MS2 (B) spectra of peak 7 in the LC-MS chromatogram of instant coffee.

To investigate the identity of the other unknown signals, the negative ionization mode was used. **Figure 4.24** shows the mass spectra for the selected signals in LC-MS analysis. It is important to indicate that the retention times of all of the compounds shifted in the LC-MS versus the HPLC-DAD analysis.

Quinic acid was assigned as the peak at 3.3 min in the HPLC chromatogram (**A** in **Figure 4.24**) by the presence of the ion $[M-H]^-$ at *m/z* 191.1 (MW = 192.17 g/mol). This result was

corroborated with HPLC-DAD by spiking the sample with the commercial standard (results not shown).

Chlorogenic acid isomers are associated with the other four peaks, three of which (**B, C** and **E** in **Figure 4.25**) show the same molecular ion peak $[M-H]^-$ = *m/z* 353 and, accordingly, were assigned to the three caffeoylquinic acid isomers (3-CQA, 4-CQA and 5-CQA) (Clifford *et al*., 2003). 5-CQA standard was already identified through comparison with the commercial standard, and assigned to the signal at 17.6 to 18.6 min (21.15 min in the HPLC chromatogram). 3-CQA was assigned based on the relative intensity of the ion fragments in the MS/MS spectra (**Figure 4.25**). Clifford *et al*. (2003) and Xie *et al*. (2011) reported the ion fragment pattern for the three caffeoylquinic acid isomers. Finally, the small and broad signal at 19 - 19.3 min (peak 11 in **Figure 4.22**) , was identified as a feruloylquinic acid isomer because it shows a parent ion $[M-H]^-$ at *m/z* 367.1, characteristic of this type of compound (Clifford *et al*., 2003).

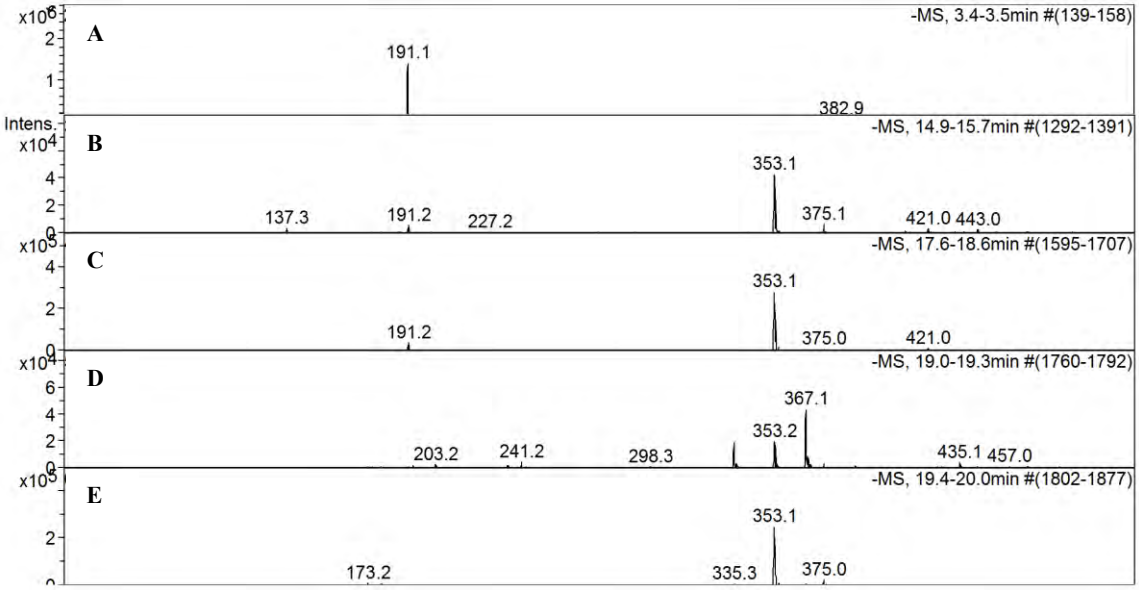


Figure 4.24. MS spectra for selected signals in the LC-MS analysis.

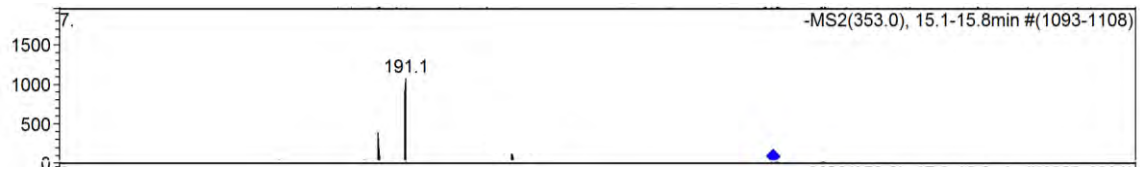


Figure 4.25. MS/MS (353.0) spectra of 3-CQA.

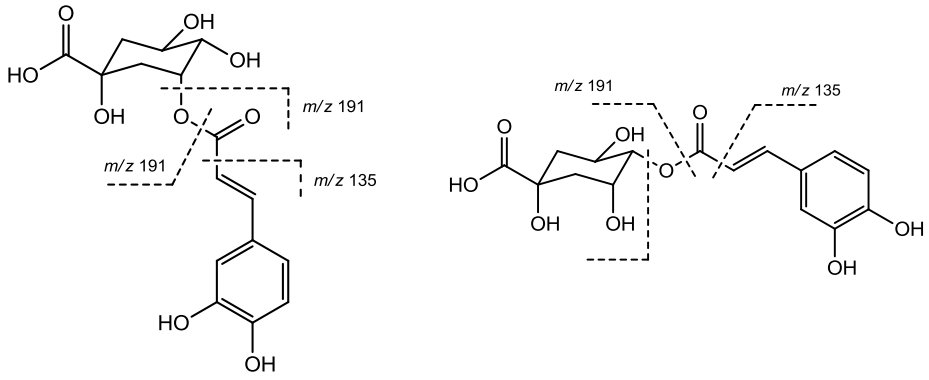


Figure 4.26. MS fragmentation pattern of 3-CQA (A) and 4-CQA (B).

In summary, the HPLC method developed allowed the simultaneous quantitation of eight compounds: caffeine, trigonelline, nicotinic acid, 1-methylpyridinium, 5-CQA, quinic acid, 3-CQA and 5-HMF. The last two compounds were not included in this study because they are not readily available in the laboratory. In the case of quinic acid the use of formic acid in the gradient system complicates its UV detection, given that both show a maximum absorption in the range from 200 to 210 nm.

4.1.1.2. HPLC-DAD quantitative analysis of coffee extracts

The HPLC quantitative study was performed in triplicate on the same six coffee extracts evaluated by NMR. The aim of this work was to validate the NMR quantitative results with classical methodology which is well-recognized as a quantitative tool: HPLC-DAD.

The amount in mg / g of each compound was calculated based on the dried mass of the aqueous coffee extracts (**Table 4.14**). The percentage errors between these results and those obtained through ^{1}H-NMR quantitation by standard calibration curves with a 9μs pulse width (PE1%), standard calibration curves with an optimized pulse width (PE2%) and ERETIC2 (PE3%) methods are summarized in **Table 4.16.** The results shown in **Table 4.16** indicate that HPLC and NMR quantitation, for trigonelline, 1-methylpyridinium and caffeine, yield for the majority of the cases, comparable results (1 – 15%). These error values are of the same magnitude as those reported in a similar study for trigonelline and caffeine quantitation in instant coffee (del Campo *et al.*, 2010*).*

The percentage error (PE%) obtained for all compounds quantitated by NMR, when compared to the validated HPLC-DAD data, indicate that they are highly dependent on the coffee sample matrix. The greatest variation observed was for caffeine. By the ERETIC2 method, values were as small as 3 %, in the case of caffeine, and as high as 25 % for instant coffee. With the ^{1}H-NMR standard calibration curves, the values for this compound, PE1%, were not better: 5 – 23 %. 1-Methylpyridinium exhibited a pronounced variation only when the ERETIC2 method was employed for quantitation (PE3%). These may be due to the inability to improve the baseline by the algorithms available in Topspin. The errors found by standard calibration curves, PE1% and PE2%, were in the range of 1 – 12 % and most of them were below 8 %. These may be the result of the baseline correction algorithm performed with the ACD software. The multiplicity of the signal monitored for the quantitation of 1-methylpyridinium (triplet) seems not to be a significant source of error.

Percentage errors for trigonelline, also a quaternary salt, are, in general, in good agreement with HPLC results, with errors below 7% in most of the cases. In contrast to 1-methylpyridinium, the errors found by the ERETIC method (PE3%), were relatively small (0.2 – 12%). This may be due to the fact that the trigonelline signal employed for quantitation is not as much affected by the baseline distortion as the 1-methylpyridinium signal. For trigonelline, ERETIC2 and the ^{1}H-NMR standard calibration curves (9μs) (2 – 12 %) gave comparable results. This behavior was also exhibited by caffeine: PE1% varied from 5 – 22% and PE3% from 3 – 25%. In the case of caffeine, the proton signal monitored was also aromatic and a sharp singlet. Caffeine content in Huayabamba coffee showed the higher errors.

As mentioned above, the results are sample dependent. The chemical environment that all these molecules are experiencing is different, hence the nature and the amount of possible complexes (caffeine adducts with chlorogenic acids, availability of different counter ions for trigonelline, 1-methylpyridinium) varies with sample, and so should the relaxation delay. This aspect should be evaluated in the future.

Similar comparisons were performed with the data available for nicotinic acid and 5-CQA. The results were not included in **Table 4.16** because, as previously foreseen, the errors were expected to be high with these compounds. They range from 50 to 150%: under-estimated for nicotinic acid and over-estimated for 5-CQA. The magnitude of the errors observed for these compounds are related to inherent matrix effects.

Table 4.14. Contents[a] of compounds 1 - 5 quantified by the HPLC-DAD method in six different types of coffee.

Coffee extract	Roasting time	Trigonelline	Nicotinic Acid	1-Methylpyridinium	Caffeine	5-Caffeoylquinic acid
Instant coffee	n.k[b]	9.313 ± 0.233	0.450 ± 0.025	1.946 ± 0.023	21.928 ± 0.337	13.457 ± 0.138
Hayabamba coffee a	t1[c]	12.094 ± 0.229	0.721 ± 0.025	3.730 ± 0.032	32.772 ± 0.089	10.483 ± 0.017
Hayabamba coffee b	t1[c] + 2min	10.078 ± 0.078	0.856 ± 0.045	4.228 ± 0.034	32.489 ± 0.161	8.429 ± 0.031
APU Espresso	t2[c] + 2min	13.356 ± 0.356	0.818 ± 0.008	4.482 ± 0.035	36.660 ± 0.217	12.604 ± 0.048
APU Gourmet	t2[c]	17.922 ± 0.190	0.636 ± 0.034	3.675 ± 0.032	36.975 ± 0.297	17.870 ± 0.170
APU Classic	t2[c]	20.014 ± 0.318	0.709 ± 0.017	4.160 ± 0.036	41.877 ± 0.239	20.028 ± 0.108

[a] Values are expressed in mg / g of ground material with n=3 for HPLC results ± standard deviation; [b] n.k = not known; [c] proprietary information

Table 4.15. Contents of compounds 1 - 5 quantified by ^{1}H-NMR as compared with the HPLC-DAD method in six different types of coffee.

Coffee	Roasting time	Trigonelline			1-Methylpyridinium			Caffeine		
		PE1[a] (%)	PE2[b] (%)	PE3[c] (%)	PE1[a] (%)	PE2[b] (%)	PE3[c] (%)	PE1[a] (%)	PE2[b] (%)	PE3[c] (%)
Instant coffee	n.k[d]	10.20	5.07	11.84	12.35	5.62	41.01	5.04	15.44	3.22
Hayabamba coffee a	t1[e]	11.99	17.92	5.12	4.53	11.38	3.35	22.72	23.84	25.98
Hayabamba coffee b	t1[e] + 2	5.96	5.90	6.51	5.26	1.67	10.08	13.71	24.09	20.39
APU Espresso	t2[e] + 2	4.94	6.45	5.62	7.60	0.17	11.96	14.56	18.16	13.87
APU Gourmet	t2[e]	3.35	8.21	6.05	7.88	2.46	22.15	8.38	13.71	3.86
APU Classic	t2[e]	2.38	14.43	0.23	7.19	9.72	18.08	5.41	17.61	5.68

[a] PE1=Percentage error between ^{1}H-NMR and HPLC calibration curve measurements (9us); [b] PE2=Percentage error between 1H-NMR and HPLC calibration curve measurements with optimized pulse width; [c] PE3=Percentage error between ERETIC2 and HPLC measurements; [d] n.k = not known; [e] proprietary information.

Figure 4.27 shows the linear regressions of the NMR standard calibration curve (9μs) (purple diamonds) and ERETIC2 results (red diamonds) versus concentrations determined by HPLC-DAD, through the entire range evaluated for trigonelline, 1-methylpyridinium and caffeine. As can be appreciated from the regression coefficient values, both above 0.9900 (0.9923 for standard calibration curves and 0.9914 for ERETIC2) overall, the agreement is highly encouraging to conclude that the NMR technique is a valuable and accurate tool for the quantitation of these three compounds in roasted coffee.

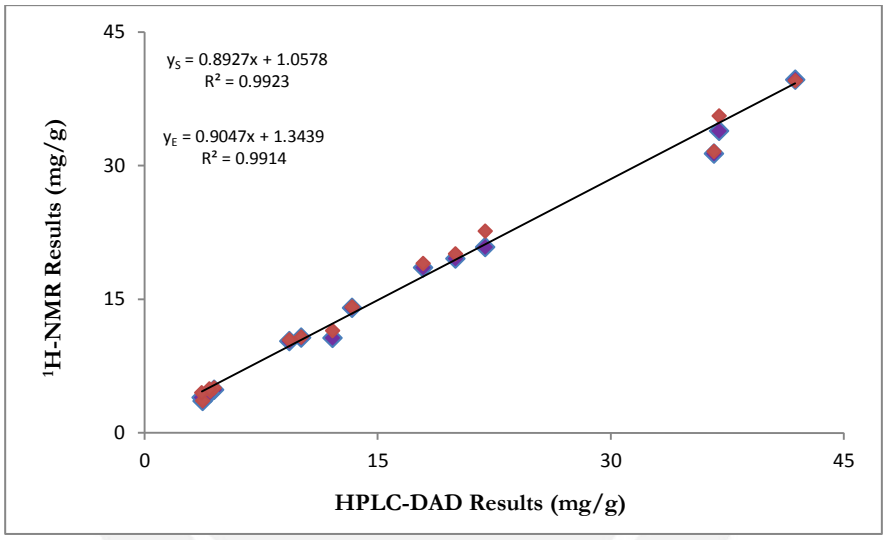


Figure 4.27. Concentrations of all coffee extracts determined by ^{1}H-NMR (standard calibration curves (purple diamonds) and ERETIC2 (red diamonds)) (Table 4.10) vs. concentration of the same coffees from HPLC-DAD (Table 4.14).

In **section 1.4.2**, the 1-methylpyridinium / trigonelline ratio was pointed as a possible roasting indicator. To test this hypothesis, the HPLC-DAD results for these two compounds were compared among the six extracts. **Table 4.16** shows the results obtained where it can be observed that both coffees with highest roasting degree, Huayabamba b and APU Espresso, show the biggest trigonelline / 1-methylpyridinium ratio. An almost equal ratio for APU Classic and APU Gourmet, despite their different quality, was also observed. These results may suggest that the trigonelline / 1-methylpyridinium ratio is independent of the quality of the bean and that it may be only related to the roasting program employed, and in consequence it may be a useful indicator to monitor the coffee

roasting degree. Further investigations have to be conducted in order to establish the usefulness of this parameter.

Table 4.16. Ratio 1-methylpyridinium / trigonelline for HPLC contents.

Compound	Roasting time	1-methylpyridinium (1-M)	Trigonelline (T)	Ratio 1-MP / T
Instant coffee	**n.k[a]**	**1.946**	**9.313**	**0.209**
Hayabamba coffee a	**t1[b]**	**3.730**	**12.094**	**0.308**
Hayabamba coffee b	**t1[b] + 2min**	**4.228**	**10.078**	**0.420**
APU Espresso	**t2[b] + 2min**	**4.482**	**13.356**	**0.336**
APU Gourmet	**t2[b]**	**3.675**	**17.922**	**0.205**
APU Classic	**t2[b]**	**4.160**	**20.014**	**0.208**

[a] n.k = not known; [b] proprietary information

In summary, the quantitation by NMR proved to be convenient for caffeine and trigonelline and 1-methylpyridinium. For nicotinic acid and 5-CQA, due to the high matrix uncontrollable effects, standard addition calibration curves need to be considered.

Finally, from the comparison of the errors found for standard calibration curves with and without pulse optimization, since there is not a significant or consistent improvement of these values, and given that the optimization is a time-consuming process, it can be concluded that a standard 9μs pulse width is sufficient to obtain quantitative results.

4.2. Principal Component Analysis (PCA)

4.2.1. PCA of ^{1}H-NMR results

Principal component analysis was used to facilitate the visualization of the NMR data through score and loading plots from which similarities and differences between the chemical compositions of the different coffee samples could be established. The idea behind the use of PCA plots is to group these samples according to roasting degrees and coffee bean qualities.

The ^{1}H-NMR spectra (0 – 10 ppm) were divided into buckets of 0.02 ppm by using AMIX Bruker software. Since a variable in the data table should define the same property over all samples, these

spectra were then aligned in fifty regular intervals using the Icoshift tool available through Matlab (Savorani *et al*., 2010). The results can be visualized in **Figure 4.28**.

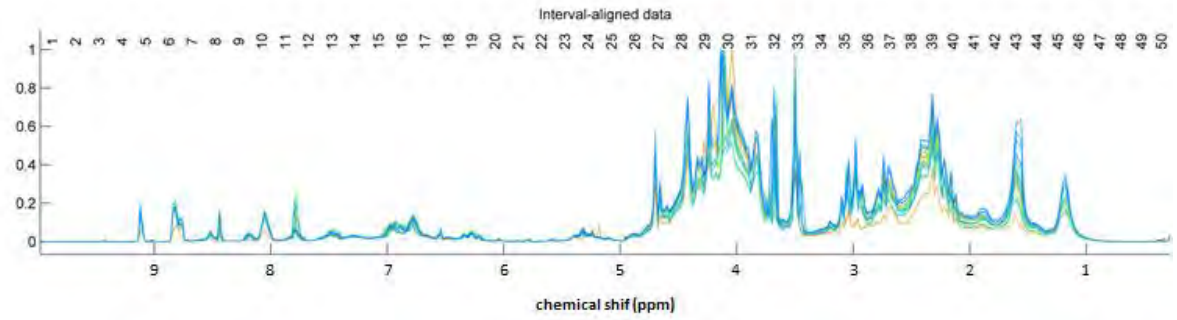


Figure 4.28. ^{1}H-NMR spectra of the coffee samples superimposed and aligned employing the Icoshift tool of MATLAB.

As can be seen in **Figure 4.28**, the region 0 to 5 ppm shows signals with much greater intensity than those within 5 to 10 ppm. Since PCA is sensitive to intensity if no scaling is performed, then major features may obscure lower intensity signals which might also have important contributions in spectra differentiation. Pareto scaling is the recommended method for the scaling of metabolomics data (Trygg *et al*., 2007) and was the chosen method for for processing the ^{1}H-NMR aligned spectra. It addresses the problem by dividing each bucket by the square root of its standard deviation, thereby up-weighting the lower intensity signals.

Figure 4.29 A shows the score plot of the two principal components, PC1 and PC2. Both components explain almost 80% of the whole spectra variability. This plot shows significant separation among the first principal component (PC1) between instant coffee (red diamonds) and specialty coffees. On the other hand, PC2 does not seem to be determining for sample differentiation. To evaluate which compounds contribute to the groupings formed through PC1, and, consequently, could be pinpointed as statistically significant metabolites, i.e. coffee markers, PC1 loadings were also analyzed (**B** in **Figure 4.29**). The upper section of the loading plot represents the metabolites that were higher in the instant coffee (located in the positive site of the PC1 axis) whereas the lower section represents metabolites that were higher in specialty coffees. The loading plot indicated 5-HMF, trigonelline, 1-methylpyridinium, formic acid, caffeine, quinic acid and lactic acid as the most influencing metabolites in the differentiation between instant and specialty coffees. A strong negative

correlation was observed between 5-HMF and the other metabolites, thus, 5-HMF was identified as responsible for the discrimination of the two sample sets. PC1 may be sufficient to correctly classify and differentiate between Peruvian roasted samples and instant coffee.

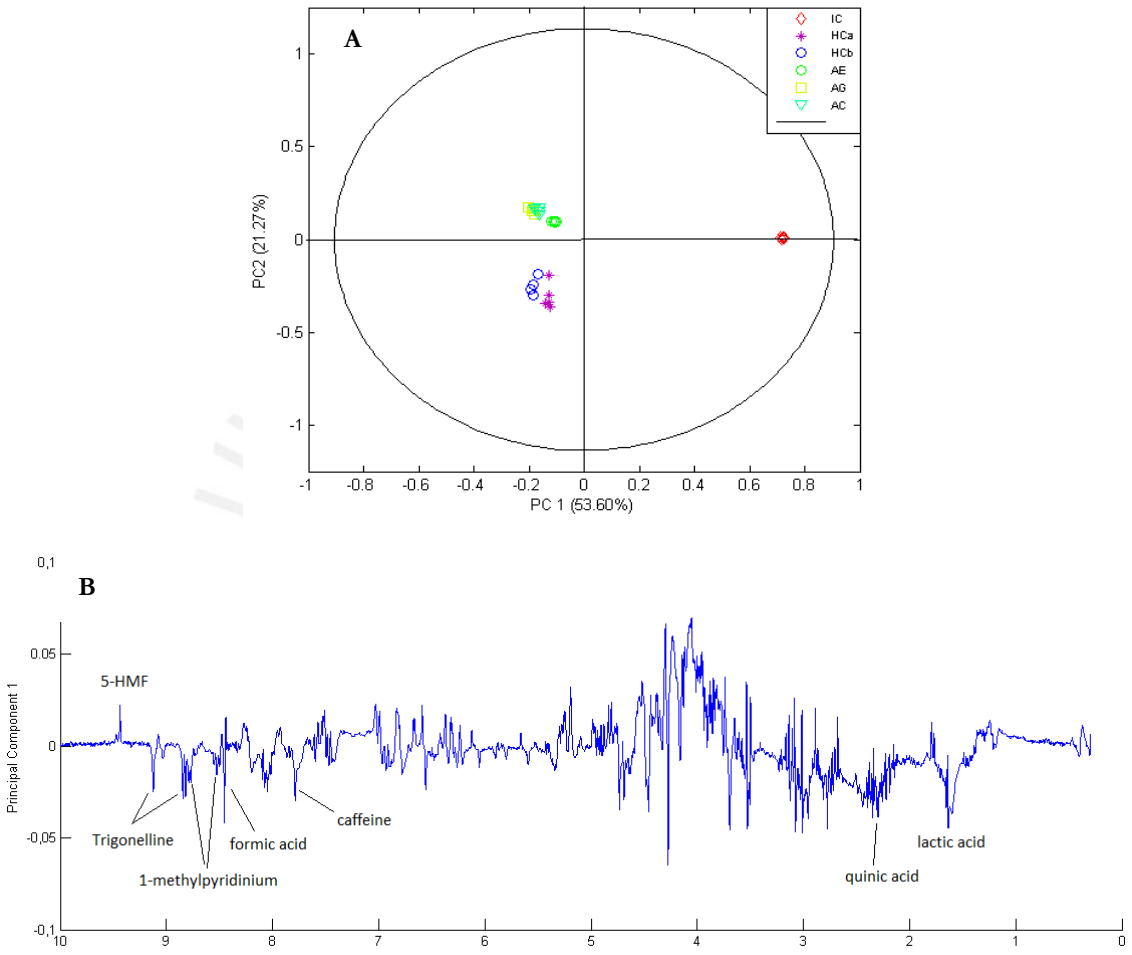


Figure 4.29. (A) Score plot of PC1 vs. PC2 for ^{1}H-NMR spectra corresponding to the six types of coffee under evaluation: IC (Instant coffee), HCa (Huayabamba coffee a), HCb (Huyabamba coffee b), AE (APU Espresso coffee), AG (APU Gourmet coffee) and AC (APU Classic coffee) (B) PC1 loading profile for the same samples.

Although discrimination between APU and Huayabamba roasted coffees can be appreciated from PC2 (**A** in **Figure 4.29**), no clear differentiation according to roasting degree was observed. As this might be due to the strong influence of 5-HMF, a new PCA was applied only to the specialty coffee data in order to investigate the metabolic differences among these coffees.

The resulting PCA score plot is shown in **Figure 4.30 A**, where, much better differentiation between specialty coffees is observed and well-defined clusters are evident. The sample from HCa (purple asterisk), closer to HCb coffee samples (blue circles) can be considered as an outlier that may be a consequence of an error in sample preparation.

APU and Huayabamba coffees, which differ between each other particularly in geographic origin (Cajamarca vs. Amazonas), are separated among the first principal component of the PCA score plot, whereas the second principal component allows a better differentiation between coffees with different roasting degrees. To further understand which compounds are responsible for this differentiation, PC1 and PC2 loadings profiles (**B** in **Figure 4.30**) were examined.

It is widely known that the geographical origin is a strong marker of the metabolic content of foods (Consonni *et al*., 2012). According to the PC1 loading profile, the features that explain the separation of Huayabamba and APU coffee are trigonelline (positive contribution), nicotinic acid and formic acid (negative contribution). These results are consistent with the NMR quantitation data (**Table 4.11**) which shows greater amounts of trigonelline for APU coffees, whereas the nicotinic acid content is higher in Huayabamba coffees. It is important to highlight here that although these coffees were grown in two close regions of Peru, Cajamarca and Amazonas (see **Figure 1.1**), a good separation was achieved, and in consequence the model may be helpful for origin predictability.

Formic acid was also identified as an important marker, however, as previously discussed, its quantification by ^{1}H-NMR requires the modification of the sample preparation protocol. PC1 also allows a better separation of APU Gourmet and APU Classic which differ in quality. According to the loading plot in **B**, this may be, principally, the result of higher levels of trigonelline in APU Classic.

As mentioned above, PC2 revealed clear separation between coffees with different roasting degrees which, as shown by the PC2 loading profile (**C** in **Figure 4.30**), is strongly influenced by trigonelline, caffeine, 1-methylpyridinium and lactic acid levels. The coffees with lower roasting times, Huayabamba coffee a (HCa), APU Gourmet (AG) and APU Classic (AC) coffees, are located in the positive side of this component whilst Huayabamba coffee b (HCb) and APU Espresso (AE) coffees were located on the negative side. These results are consistent with the NMR quantitation results (**Table 4.11**) and literature data, according to which, 1-methylpyridinium is one of the

principal thermal degradation products of trigonelline, and, in consequence, its concentration is increased by longer roasting times, whereas that of trigonelline decreases.

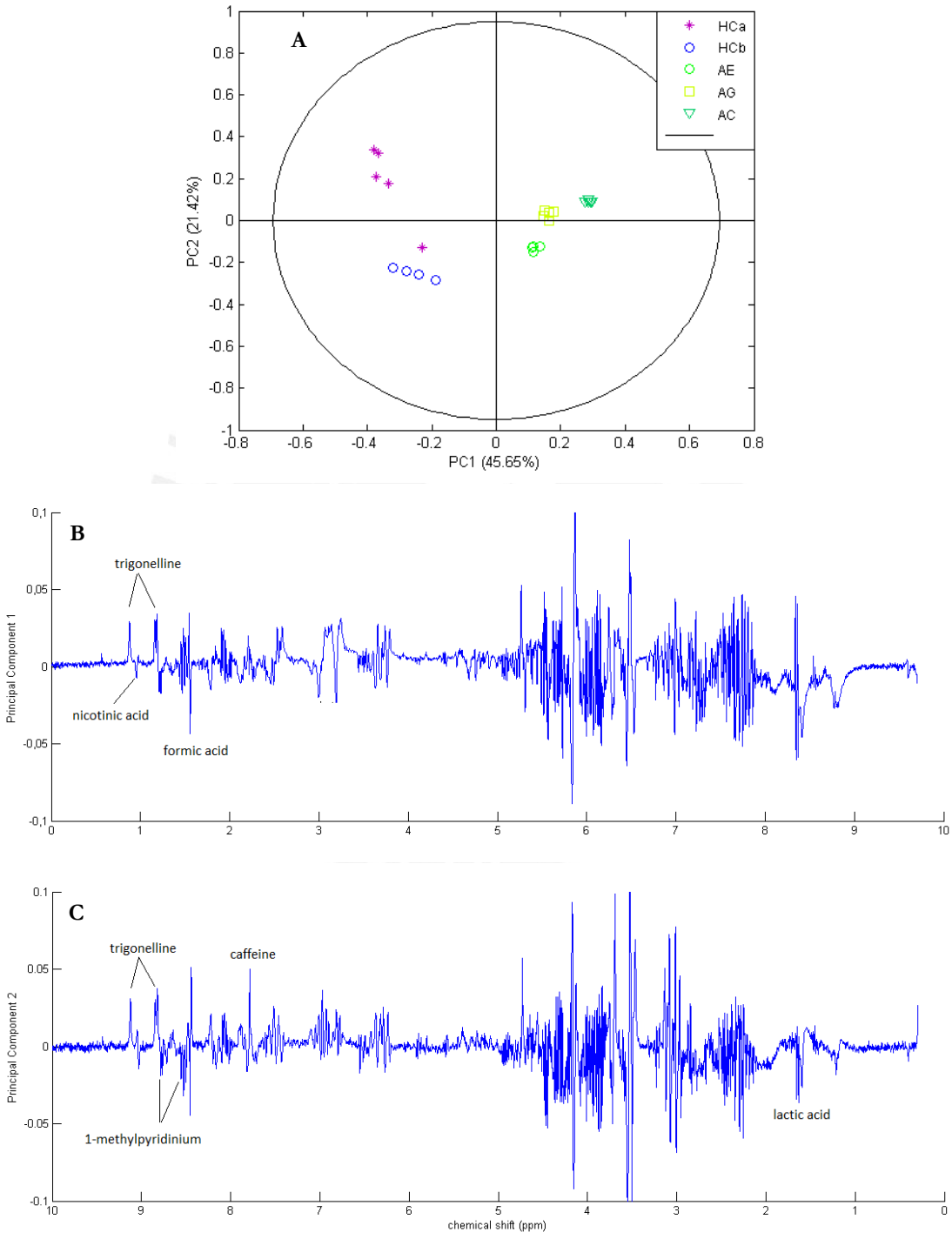


Figure 4.30. (A) Score plot of PC1 vs. PC2 for ^{1}H-NMR spectra corresponding to the five Peruvian specialty coffee samples under study. (B) PC1 and (C) PC2 loading profiles for the same samples.

Lactic and quinic acid were also located in the lower section of the loading profile, which means that their concentration may increase during roasting. The increase in lactic acid levels with longer roasting times was previously reported by Wei *et al.* (Wei *et al.*, 2012). In the case of quinic acid, it is well-known that its concentration increases in the roasted bean as a consequence of the chlorogenic acid cleavage during roasting, which releases the quinic acid moiety (Clarke and Vitzthum, 2001, Blumberg *et al*., 2010).

In summary, both PCA results suggest that trigonelline, 1-methylpyridinium, caffeine and nicotinic acid, all of them quantified by HPLC-DAD and NMR in the present study, are important markers that have a major influence in the statistical differentiation of different populations, and could be useful for coffee classification as a function of quality and roasting.

4.2.2. PCA of HPLC results

A principal component analysis (PCA) was performed on the HPLC quantitation results (**Table 4.11**) taking into account the areas and concentrations of the compounds evaluated in the different coffee extracts. Pareto scaling was applied prior to PCA analysis with the aim to balance the contribution of each compound to the overall variance, given the wide range of concentrations between them.

The loading and score plot profiles corresponding to the first two principal components, which explain almost 100% of the total variance, are displayed in **Figure 4.31**. It is important to mention that HPLC PCA analysis does not consider the whole variability among the samples as in the case of NMR, instead, the measure of the variability is done only regarding the compounds that were quantified by this method, compounds **1** – **5** (**Table 4.14**). Previously, the results of the principal component treatment of the NMR spectra (**Figure 4.30**) suggested that compounds **1** – **4** may be enough to achieve a successful differentiation among the different coffees. This assumption was confirmed by the results shown in the PCA score plot (**B** in **Figure 31)**, where good separation and clustering of the coffee samples is observed. In the biplot (**A** in **Figure 4.31**) the different compounds are projected in the plane of the score plot and in that way it can appreciated how, and to what extent, the different directions in the score plot are influenced by them.

As in the case of the ^{1}H-NMR chemometric results, it becomes clear that the first principal component which explains almost 80 % of the total variability represents a strong positive correlation between caffeine and trigonelline. However in contrast to the loading plot of ^{1}H-NMR (**Figures 4.29** and **4.30**), 5-CQA shows a significant influence in coffee differentiation both, among PC2 and PC1, whereas the contributions of 1-methylpyridinium and nicotinic acid are less significant.

As mentioned above, the distribution along the first principal component is strongly affected by caffeine (Caf), trigonelline (Trig) and 5-CQA and to a lesser extent by the 1-methylpyridinium ion and nicotinic acid, all of them positively correlated along PC1. APU coffees and instant / Huayabamba coffees are oppositely placed with respect to the vertical axis, which means that they have a negative correlation, particularly regarding to 5-CQA, caffeine and trigonelline (the most influencing variables). This is in agreement with the results shown in **Table 4.15**, where it can be seen that APU coffees have significant higher contents of 5-CQA, trigonelline and caffeine, especially in the case of APU Gourmet (light green squares) and APU Classic (green triangles), both clearly more displaced to the right. As expected from the results in **Table 4.15**, the influence of 1-methylpyridinium and nicotinic acid is not critical for Huayabamba and APU coffee differentiation, which means that their variability is not closely related to quality and geographical origin.

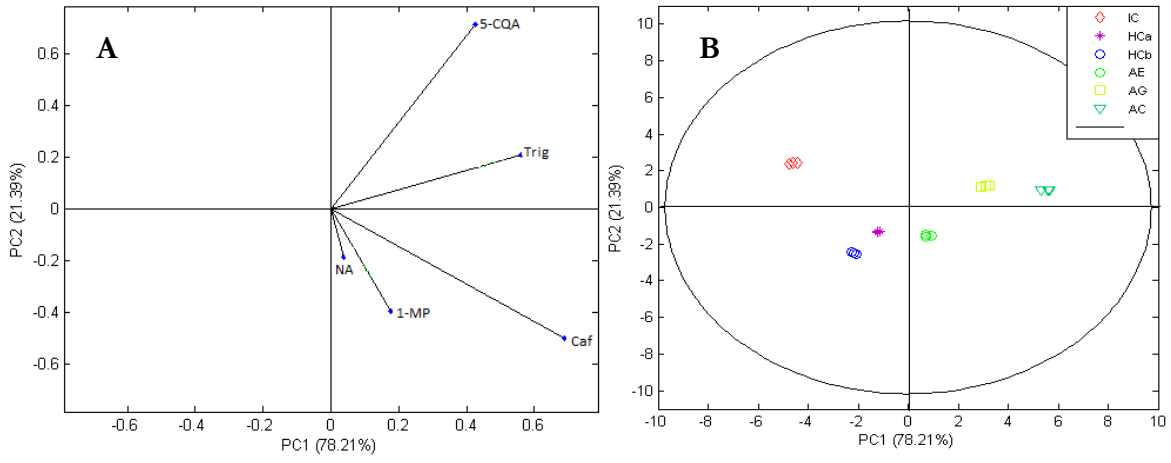


Figure 4.31. PCA score plot of PC1 vs. PC2 (A) and biplot (B) corresponding to HPLC quantitation results.

The second principal component which explains roughly 20 % of the data allows the differentiation between coffees with different roasting degree. As can be seen in the loading plot APU Gourmet and APU Classic are located in the upper site of the horizontal axis whereas APU Espresso (green circles) and Huayabamba coffees are located in the lower side. AG and AC have the same roasting time and are located in almost in the same position with respect to PC2, and Huayabamba coffee b, which have a longer roasting time than Huayabamba coffee a, is located below of it. These results suggest that APU Espresso and HCa may have similar grades of roasting, and that instant coffee may have the lowest roasting degree. To evaluate which compounds are responsible for this distribution, the loading plot must be examined. In contrast to PC1, a negative correlation between caffeine and 5-CQA / trigonelline is observed. Additionally, 1-methylpyridinium and nicotinic acid contribute in a higher extent to the coffee discrimination than trigonelline, to which they are negatively correlated. This is in agreement with the reported thermal degradation of trigonelline during roasting into 1-methylpyridinium and nicotinic acid, and with the quantitative HPLC-DAD results (**Table 4.15**) which show a higher content of both compounds in the coffees with the longer roasting times, Huayabamba coffee b and APU Espresso.

In the present study, different coffee types were investigated according to their metabolic differences demonstrating effects of roasting time, quality and geographical origin, on the metabolite composition of coffee. It was demonstrated using two different methodologies, NMR and HPLC, coupled with multivariate statistical analysis that the chemical profile of the coffee extracts is strongly affected by variation in the content of compounds such as trigonelline, 5-HMF, nicotinic acid, formic acid, caffeine, 1-methylpyridinium, quinic acid and lactic acid. This analytical approach may be a useful and objective tool for understanding and tracing the metabolic profile variation of coffee during roasting in order to define appropriate roasting times and temperatures for the process. Additionally, it may be helpful to determine the geographic origin and reproducibility of production with the aim to guarantee a good and consistent quality of the final product.

5. CONCLUSIONS AND FUTURE WORK

A new ^{1}H-NMR quantitative protocol was developed for the simultaneous quantitation of trigonelline, caffeine, nicotinic acid, 1-methylpyridinium and 5-caffeoylquinic acid in various types of Peruvian specialty coffees. Unlike the NMR quantitative method available in the literature for the quantitation of these compounds, which employed ^{13}C-NMR signals for quantitation, requiring longer acquisition times and the use of relaxing agents, the method developed in the present investigation was performed in only 11 min.

Two methods were evaluated and compared for quantitative ^{1}H-NMR: ERETIC2 and standard calibration curves. The percentage differences between these methodologies were in the range of 1 % to 15 % in more than 75 % of the cases. The differences varied with sample. The precision (n=5) of both methods was very high (small standard deviations).

The HPLC methodology developed constitutes the first validated method for the simultaneous quantitation of trigonelline, caffeine, nicotinic acid, 1-methylpyridinium and 5-caffeoylquinic acid. The linear regression parameters indicate a good linear correlation, LOD values and LOQ values which indicate good sensitivity. The reproducibility, verified by intra-day and inter-day repetitive analysis, also showed satisfactory results. The ^{1}H-NMR quantitative methodology developed was compared with the HPLC-DAD validated method, obtaining percentages errors below 15 % (in the majority of the cases), for trigonelline, 1-methylpyridinium and caffeine, when standard calibration curves where employed as the quantitative tool and below 20 % when ERETIC2 was used.

The errors for nicotinic acid and 5-caffeoylquinic acid were very high compared to the results obtained by HPLC. Standard addition calibration curves for the quantitation of these two compounds are recommended.

The great variability in the percentage errors among samples between HPLC and the quantitative NMR methodologies is associated with matrix effects. The optimization of the 90° pulse width of T_1 for each type of coffee and metabolite analyzed does not significantly improve the errors.

The Principal Component Analysis allowed the distinction among the various coffee beans studied, based on origin and roasting conditions. Trigonelline, 1-methylpyridinium, caffeine, 5-caffeoylquinic acid, 5-hydroxymethylfurfural, nicotinic acid, quinic acid, formic acid and lactic acid, seemed to be determinant for the clustering observed with major influence in the statistical differentiation of different populations and may be useful for classification as a function of quality and roasting. 5-Hydroxymethylfurfural was identified as the principle responsible for the differentiation between Peruvian specialty coffees and instant coffee.

The HPLC method developed allowed the separation of eight compounds: caffeine, trigonelline, nicotinic acid, 1-methylpyridinium, 5-CQA, quinic acid, 3-CQA and 5-HMF. The last three compounds were confirmed by LC-MS-MS analysis. Additionally, the ratio of 1-methylpyridinium / trigonelline was calculated and proven to be a useful measurement of the roasting process, independent of the quality of the roasted bean.

Formic acid, reported as an important contributor to coffee acidity and identified in the present study as a roasting marker from the PCA analysis, was also identified as a non-overlapped signal in NMR suitable for quantitation, but it was not quantitated in this study because its concentration was affected during lyophilization. Further work needs to be conducted in order to quantitate the volatile acids such as formic and acetic acid. Direct D_2O extraction is recommended.

It was demonstrated that the ^{1}H-NMR and HPLC-DAD methodology developed could be used as a tool to optimize the roasting conditions of the different specialty coffees available in CENFROCAFE. Further studies must be done in order to relate the metabolic profile of the coffees with cup score values.

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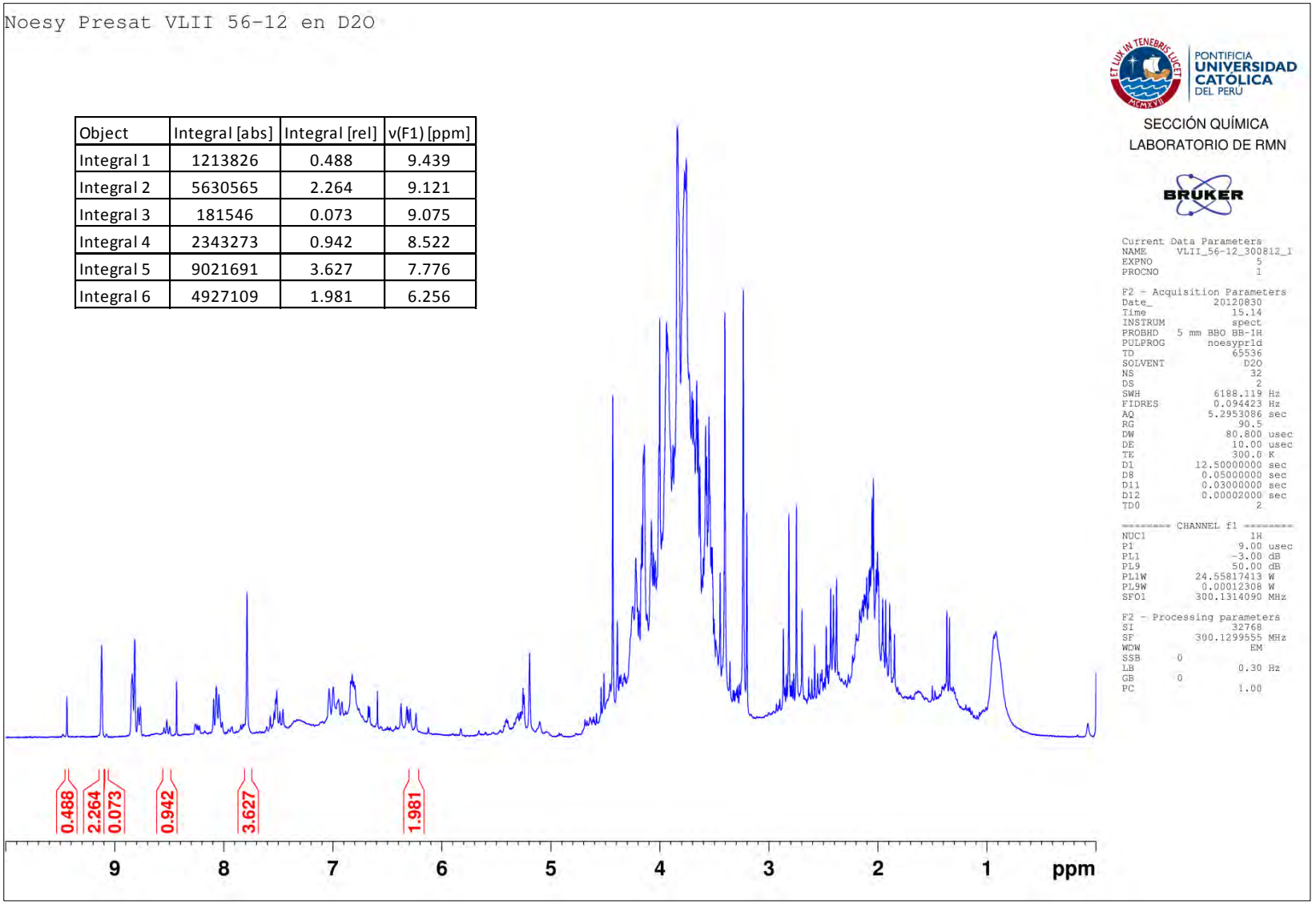
7. ANNEXES

ANNEX 1. ^{1}H-NMR spectrum of an instant coffee sample processed with Topspin 2.1.

Noesy Presat VLII 56-12 en D2O

Object	Integral [abs]	Integral [rel]	v(F1) [ppm]
Integral 1	1213826	0.488	9.439
Integral 2	5630565	2.264	9.121
Integral 3	181546	0.073	9.075
Integral 4	2343273	0.942	8.522
Integral 5	9021691	3.627	7.776
Integral 6	4927109	1.981	6.256



PONTIFICIA UNIVERSIDAD CATÓLICA DEL PERÚ

SECCIÓN QUÍMICA
LABORATORIO DE RMN

BRUKER

```
Current Data Parameters
NAME      VLII_56-12_300812_1
EXPNO                       5
PROCNO                      1

F2 - Acquisition Parameters
Date_              20120830
Time                  15.14
INSTRUM                spect
PROBHD   5 mm BBO BB-1H
PULPROG            noesypr1d
TD                     65536
SOLVENT                  D2O
NS                        32
DS                         2
SWH                6188.119 Hz
FIDRES            0.094423 Hz
AQ                5.2953086 sec
RG                      90.5
DW                  80.800 usec
DE                  10.00 usec
TE                    300.0 K
D1               12.50000000 sec
D8                0.05000000 sec
D11               0.03000000 sec
D12               0.00002000 sec
TD0                        2

======== CHANNEL f1 ========
NUC1                      1H
P1                     9.00 usec
PL1                   -3.00 dB
PL9                   50.00 dB
PL1W             24.55817413 W
PL9W              0.00012308 W
SFO1             300.1314090 MHz

F2 - Processing parameters
SI                     32768
SF               300.1299555 MHz
WDW                       EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
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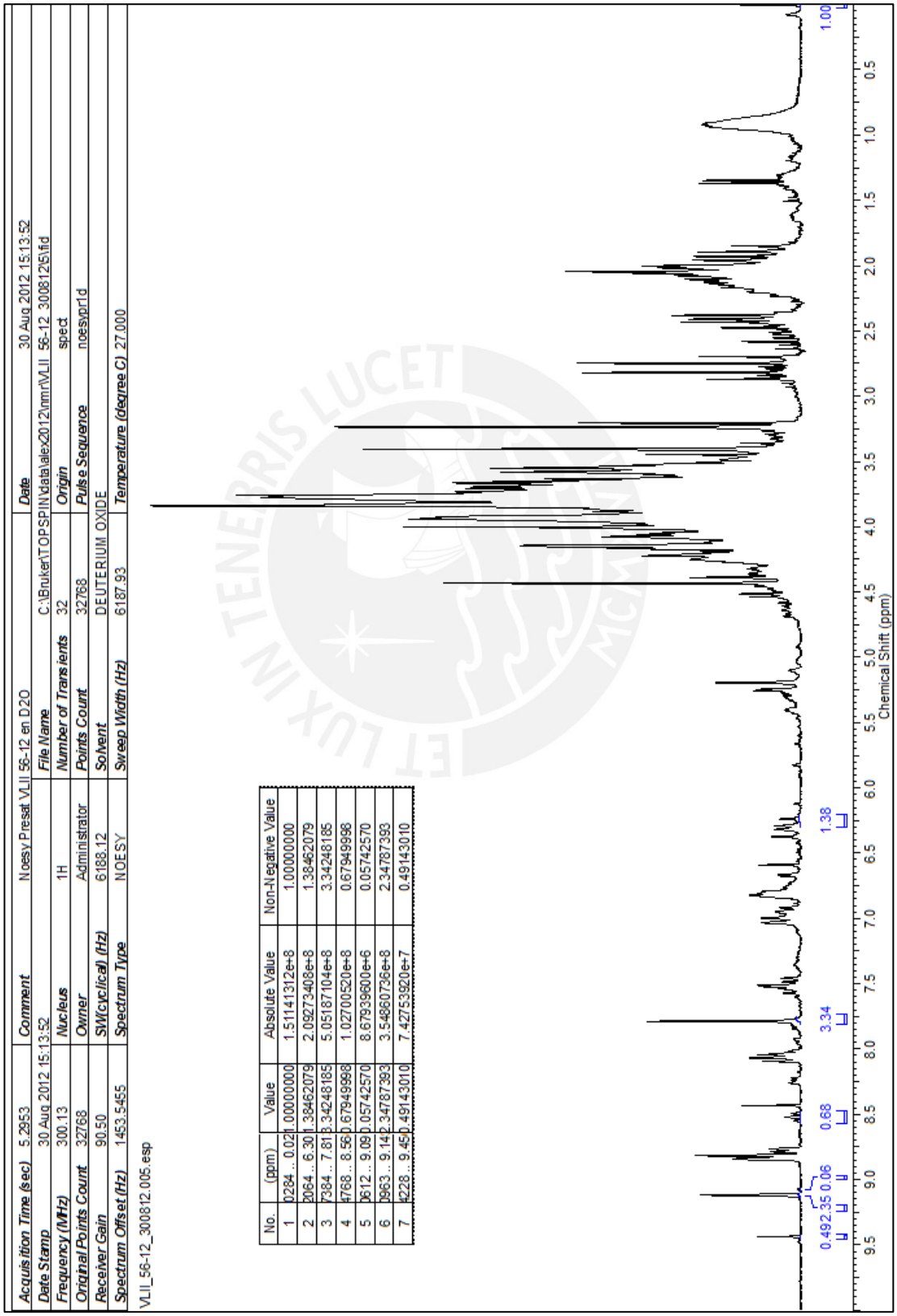
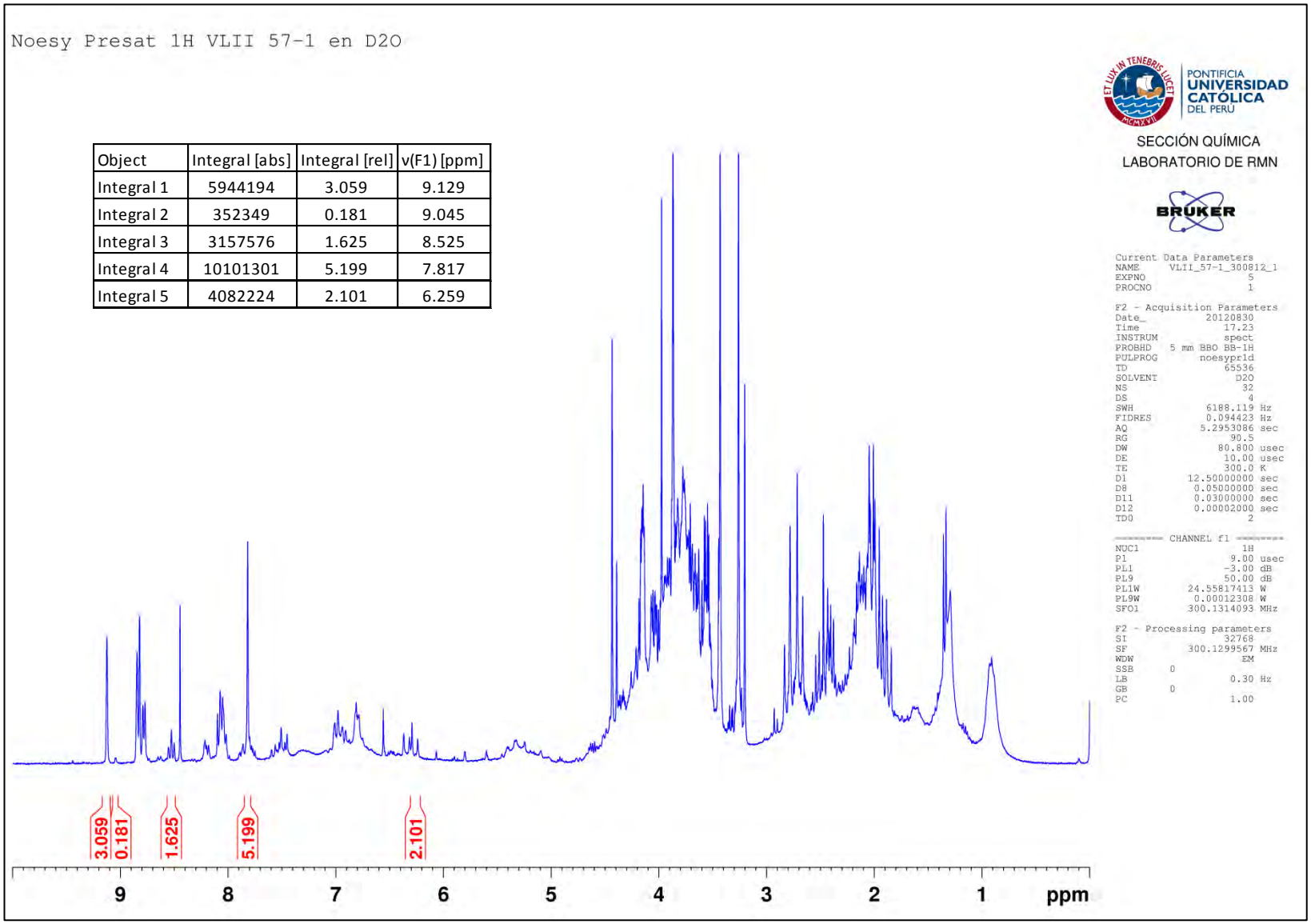
ANNEX 2. ^{1}H-NMR spectrum of an instant coffee sample processed with ACD 12.01.

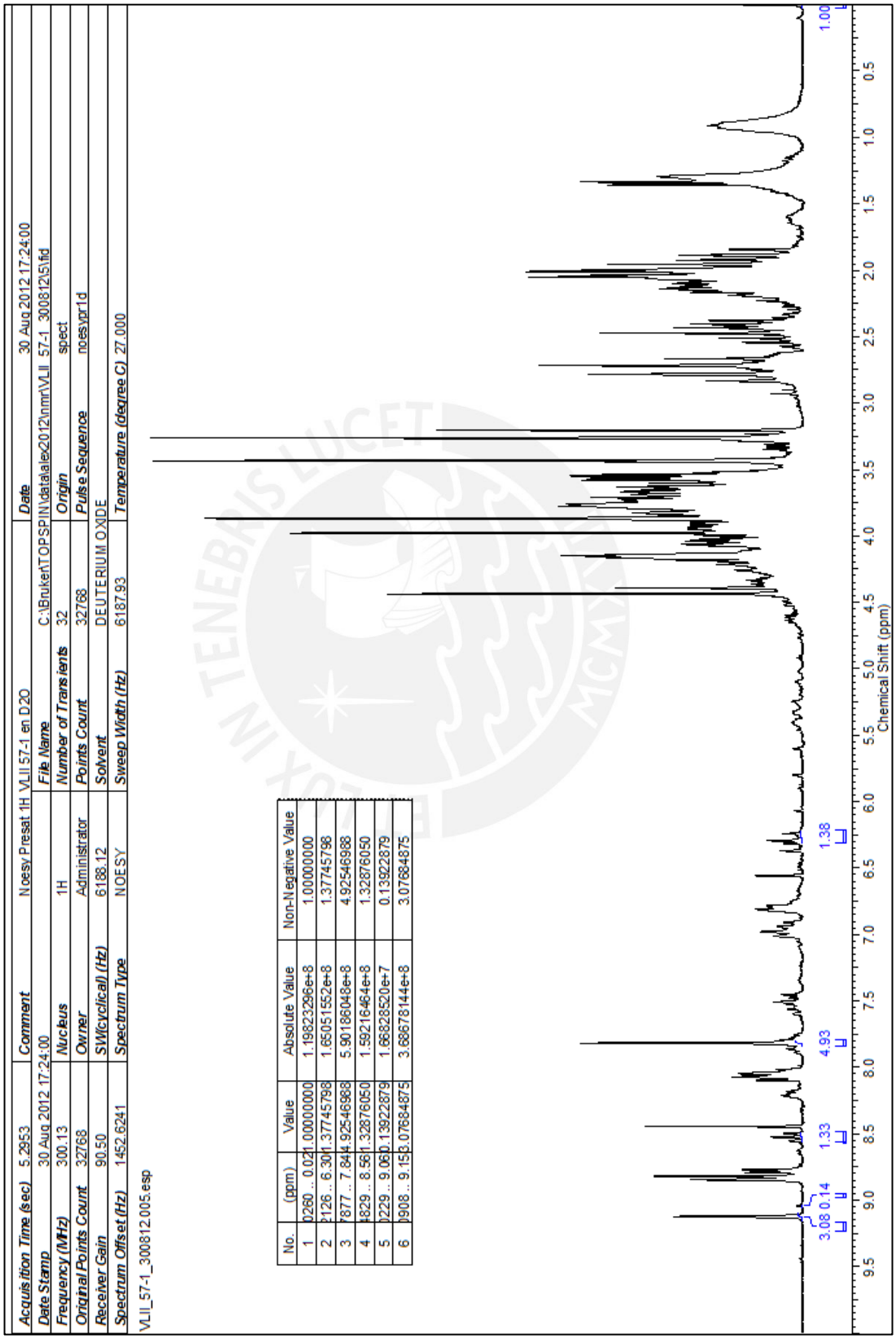


ANNEX 3. ^{1}H-NMR spectrum of a Huayabamba coffee a sample processed with Topspin 2.1.

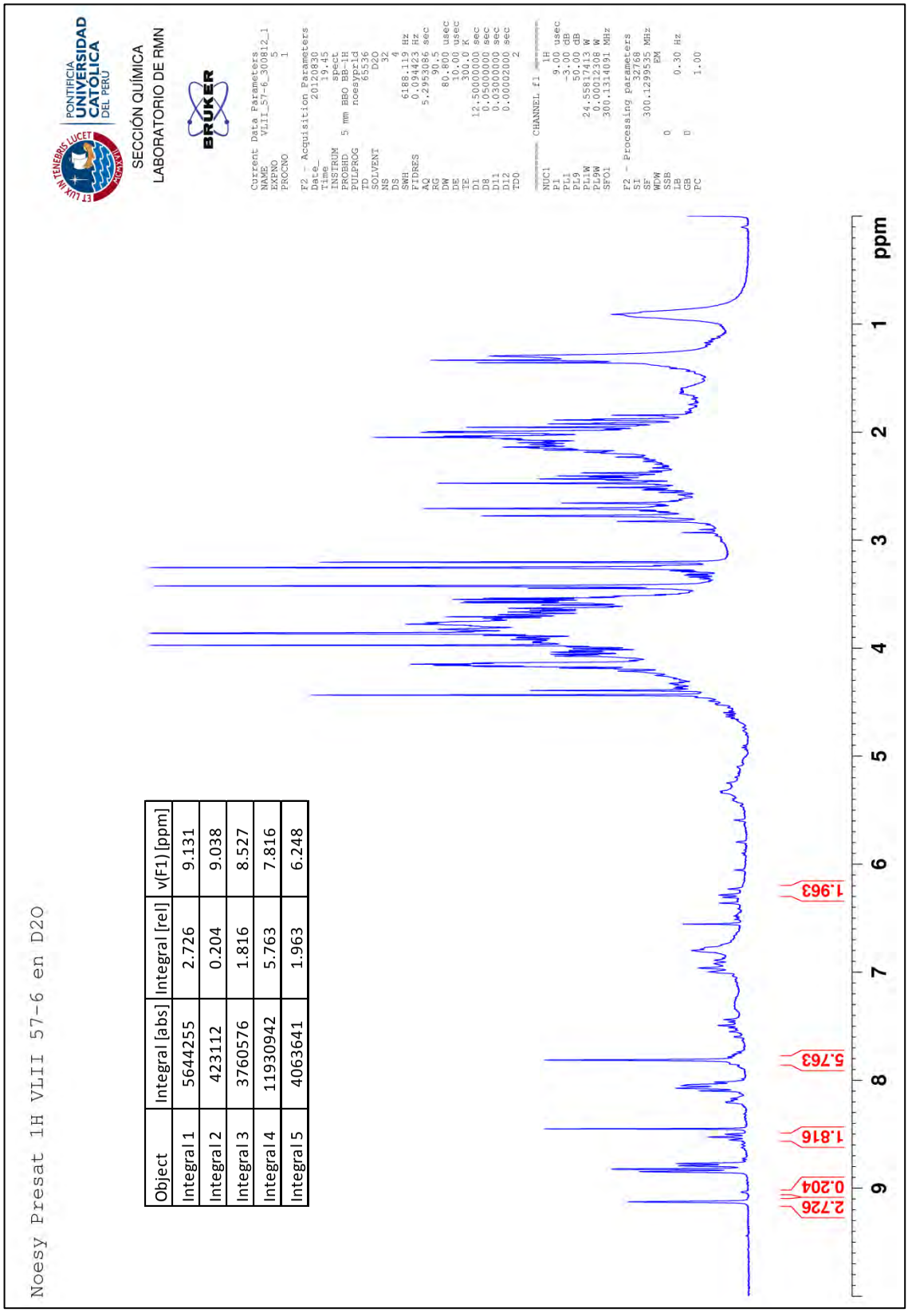


Object	Integral [abs]	Integral [rel]	v(F1) [ppm]
Integral 1	5944194	3.059	9.129
Integral 2	352349	0.181	9.045
Integral 3	3157576	1.625	8.525
Integral 4	10101301	5.199	7.817
Integral 5	4082224	2.101	6.259

ANNEX 4. ^{1}H-NMR spectrum of a Huayabamba coffee a sample processed with ACD 12.01.

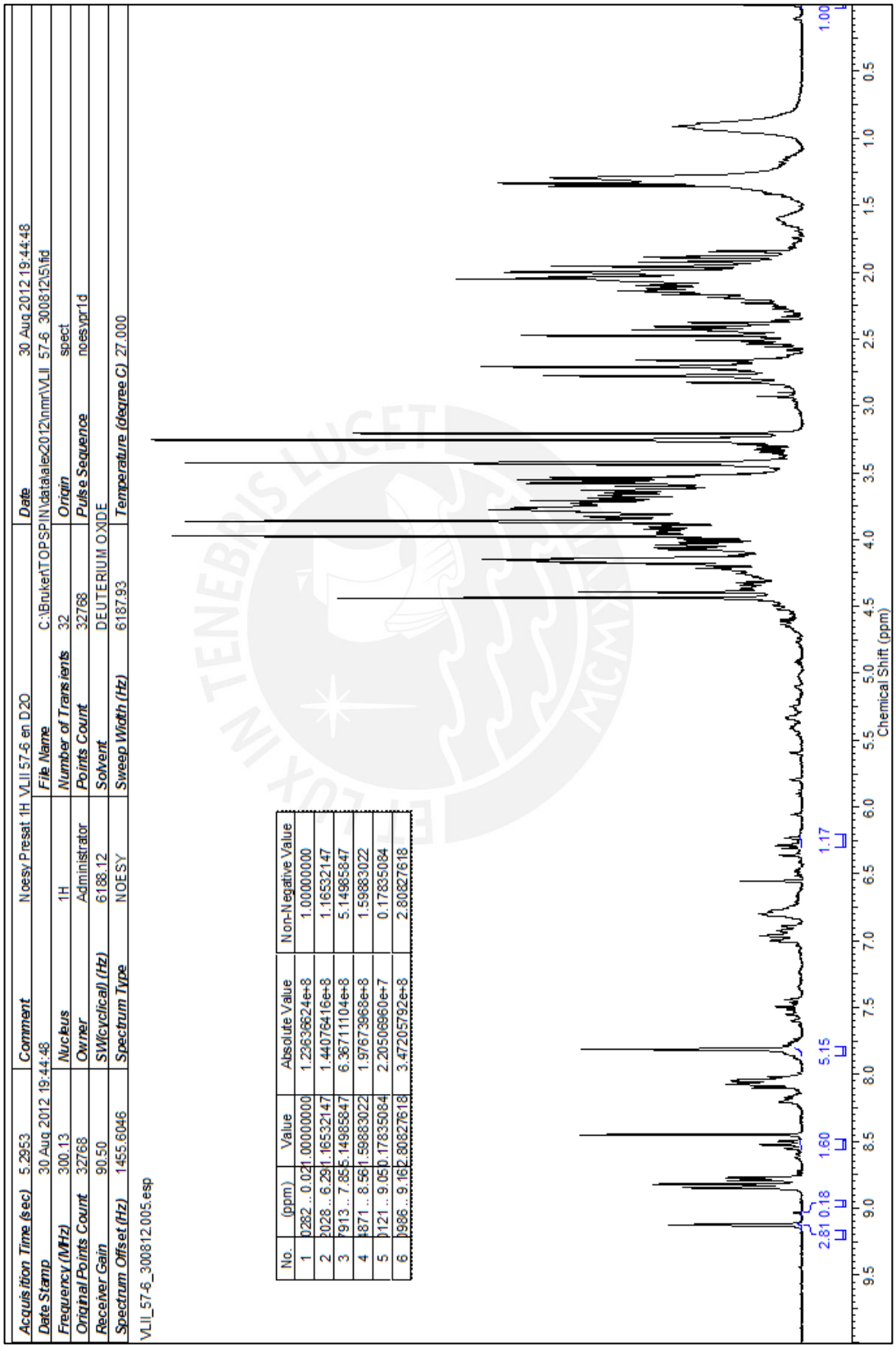


ANNEX 5. ^{1}H-NMR spectrum of a Huayabamba coffee b sample processed with Topspin 2.1.

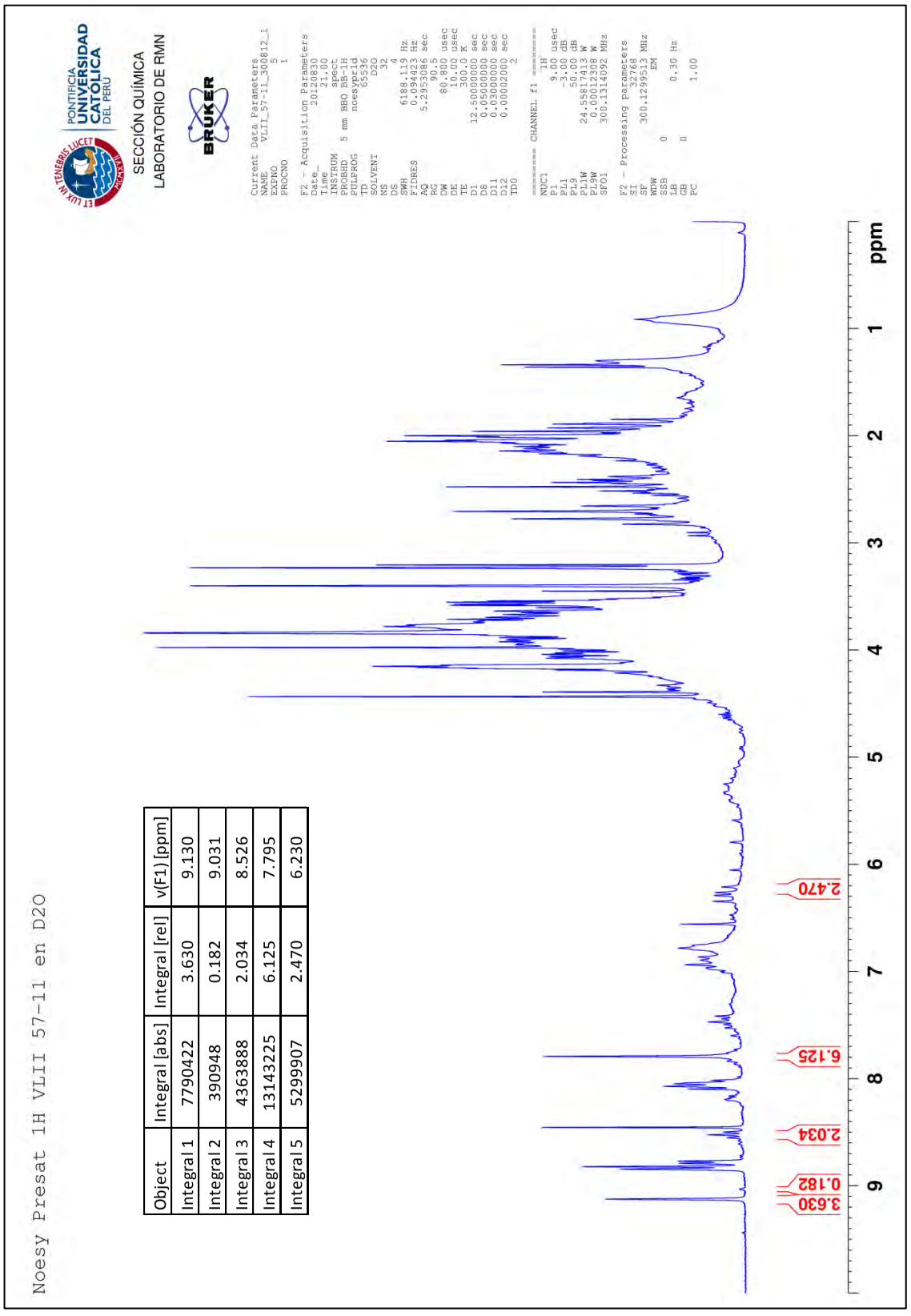


Object	Integral [abs]	Integral [rel]	v(F1) [ppm]
Integral 1	5644255	2.726	9.131
Integral 2	423112	0.204	9.038
Integral 3	3760576	1.816	8.527
Integral 4	11930942	5.763	7.816
Integral 5	4063641	1.963	6.248

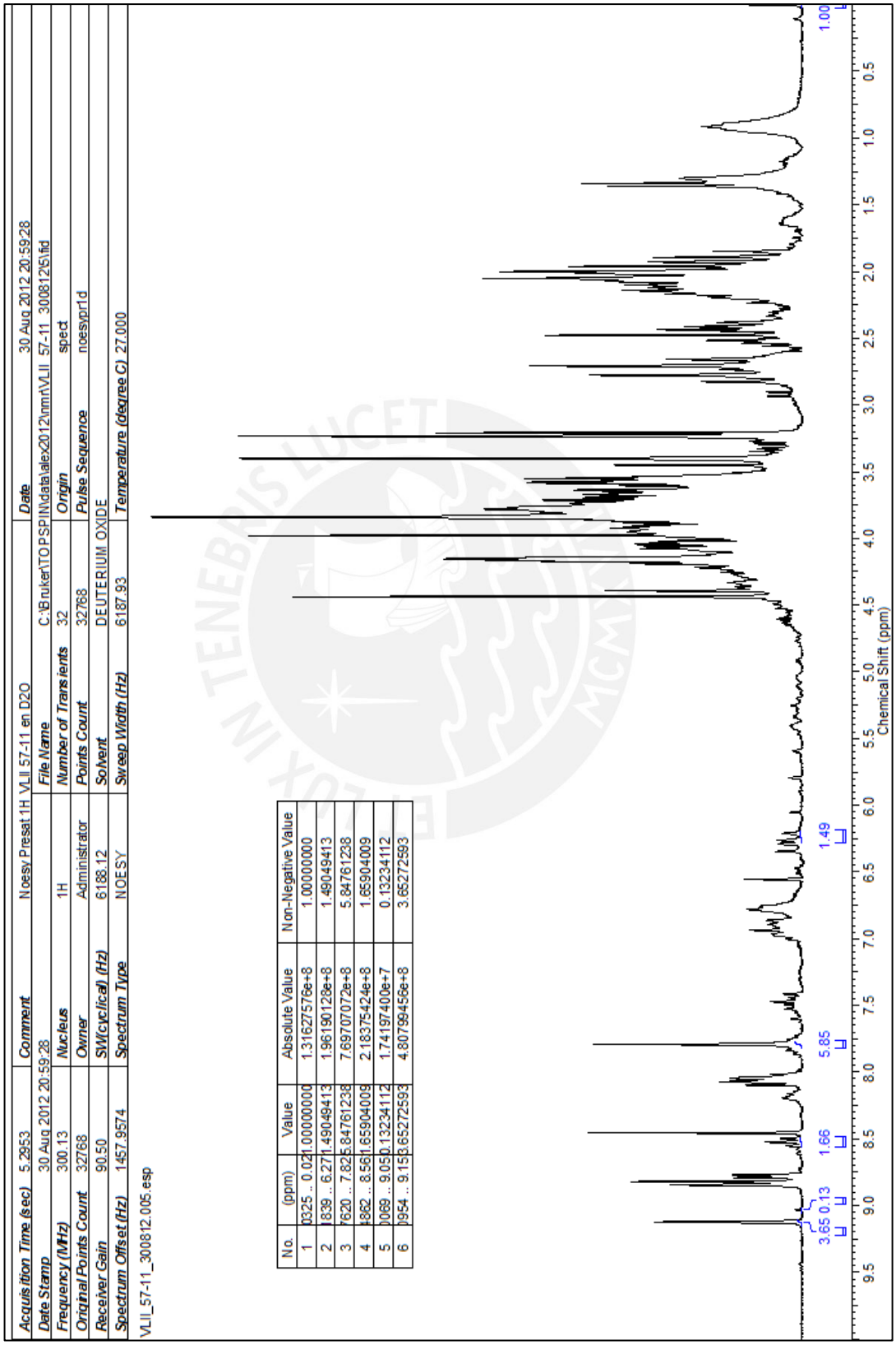
ANNEX 6. ^{1}H-NMR spectrum of a Huayabamba coffee b sample processed with ACD 12.01.



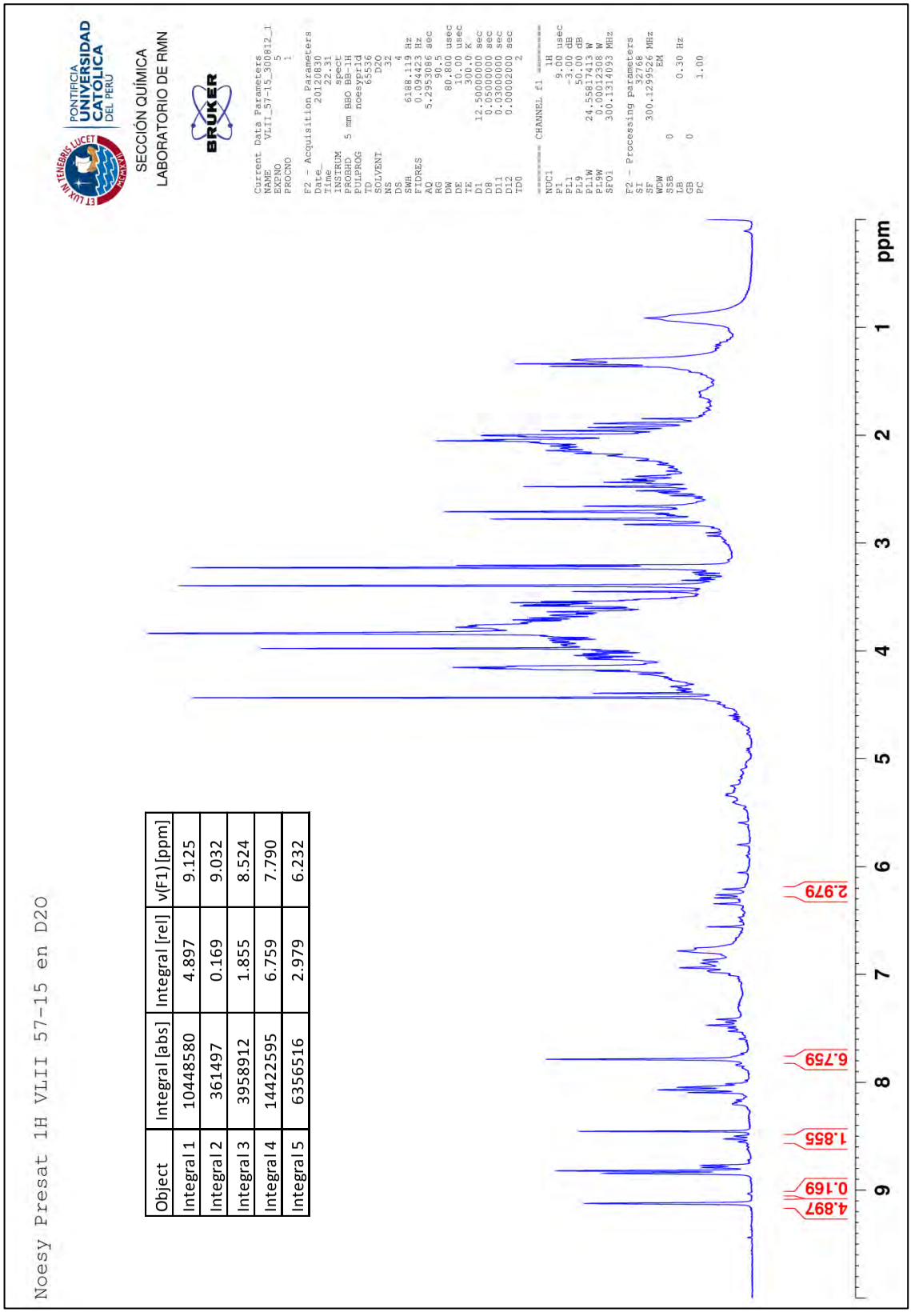
ANNEX 7. ^{1}H-NMR spectrum of an APU Espresso coffee sample processed with Topspin 2.1.



ANNEX 8. ^{1}H-NMR spectrum of an APU Espresso coffee sample processed with ACD 12.01.

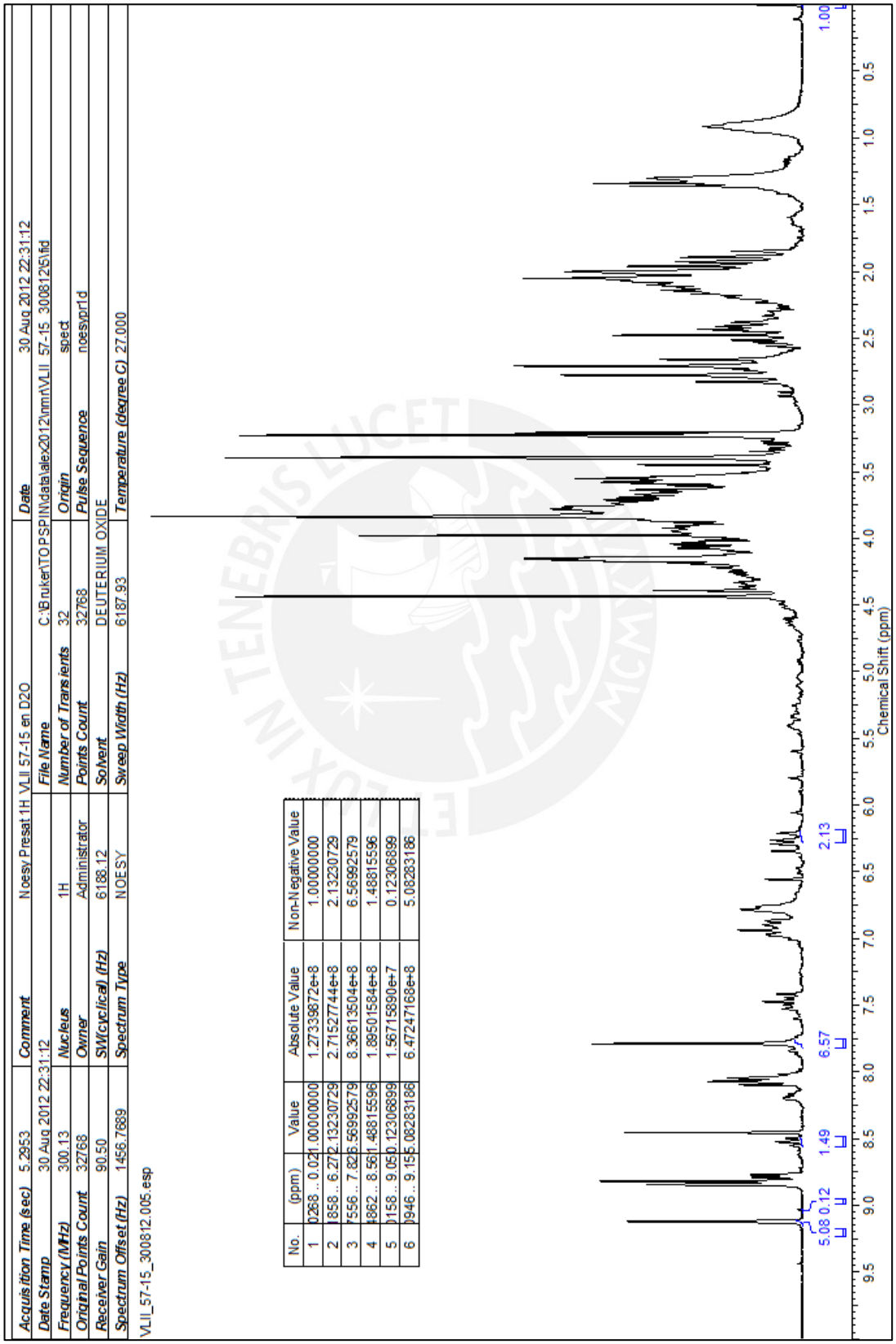


ANNEX 9. ^{1}H-NMR spectrum of an APU Gourmet coffee sample processed with Topspin 2.1.

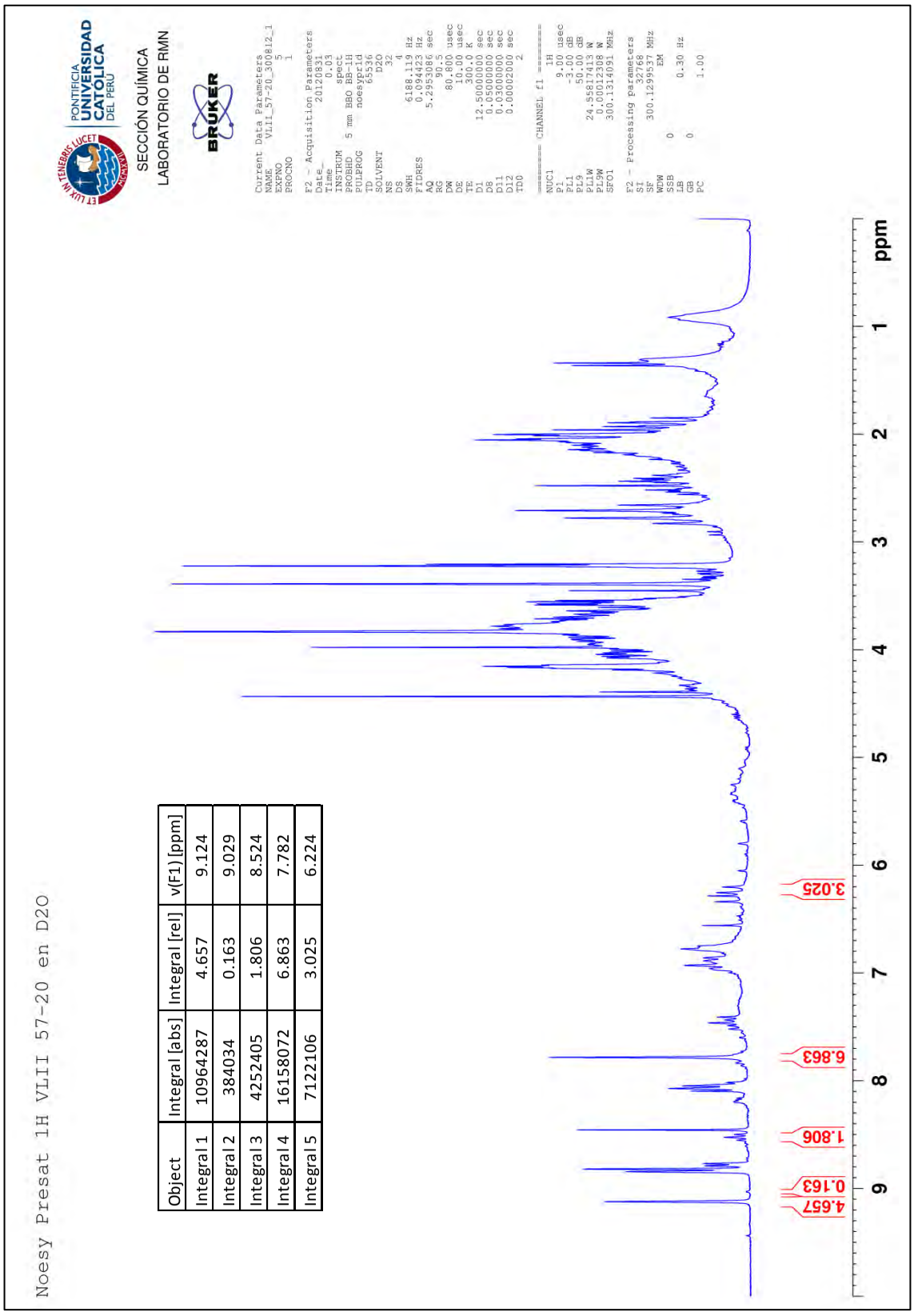


Object	Integral [abs]	Integral [rel]	v(F1) [ppm]
Integral 1	10448580	4.897	9.125
Integral 2	361497	0.169	9.032
Integral 3	3958912	1.855	8.524
Integral 4	14422595	6.759	7.790
Integral 5	6356516	2.979	6.232

ANNEX 10. ^{1}H-NMR spectrum of an APU Gourmet coffee sample processed with ACD 12.01.

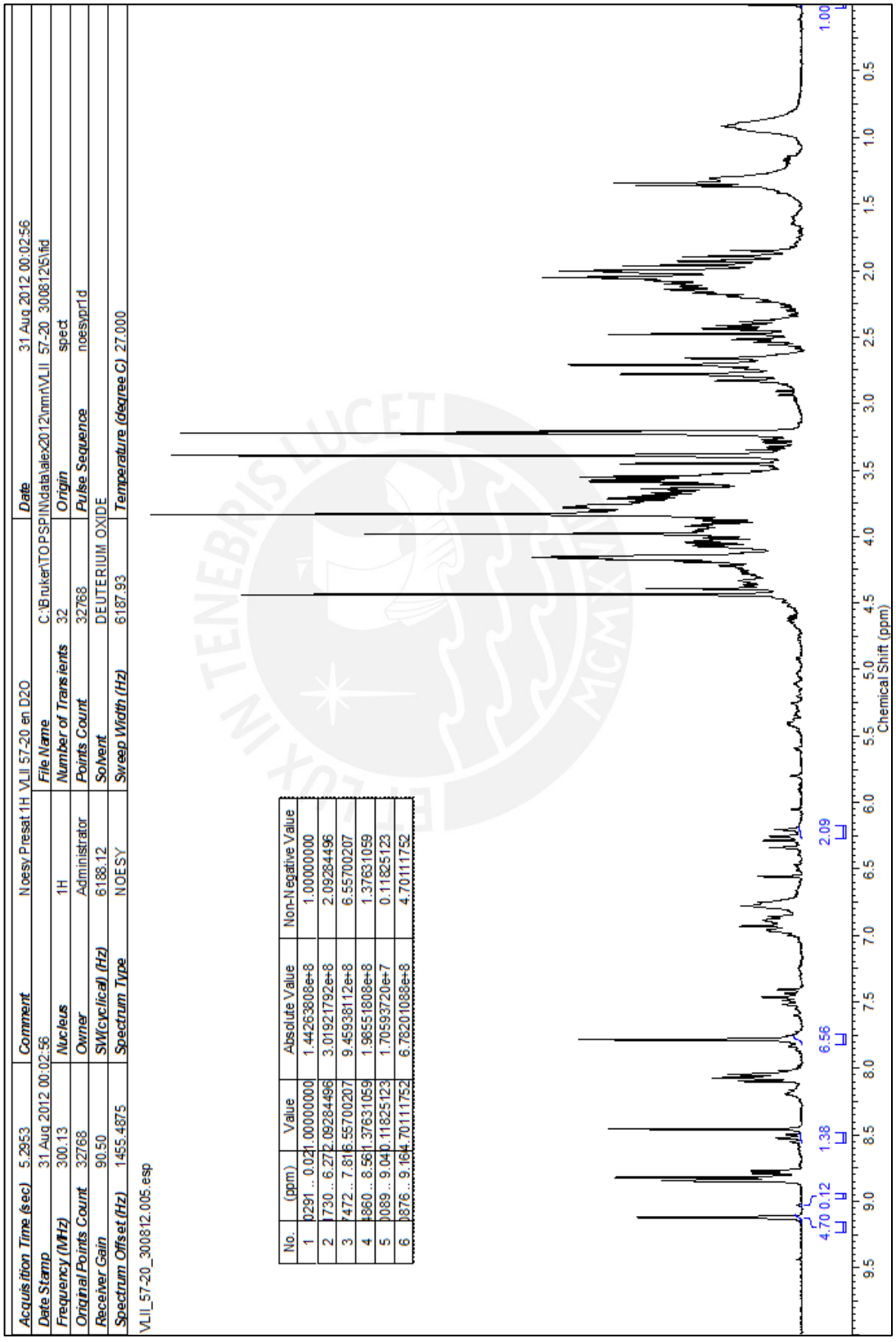


ANNEX 11. ^{1}H-NMR spectrum of an APU Classic coffee sample processed with Topspin 2.1.



Object	Integral [abs]	Integral [rel]	v(F1) [ppm]
Integral 1	10964287	4.657	9.124
Integral 2	384034	0.163	9.029
Integral 3	4252405	1.806	8.524
Integral 4	16158072	6.863	7.782
Integral 5	7122106	3.025	6.224

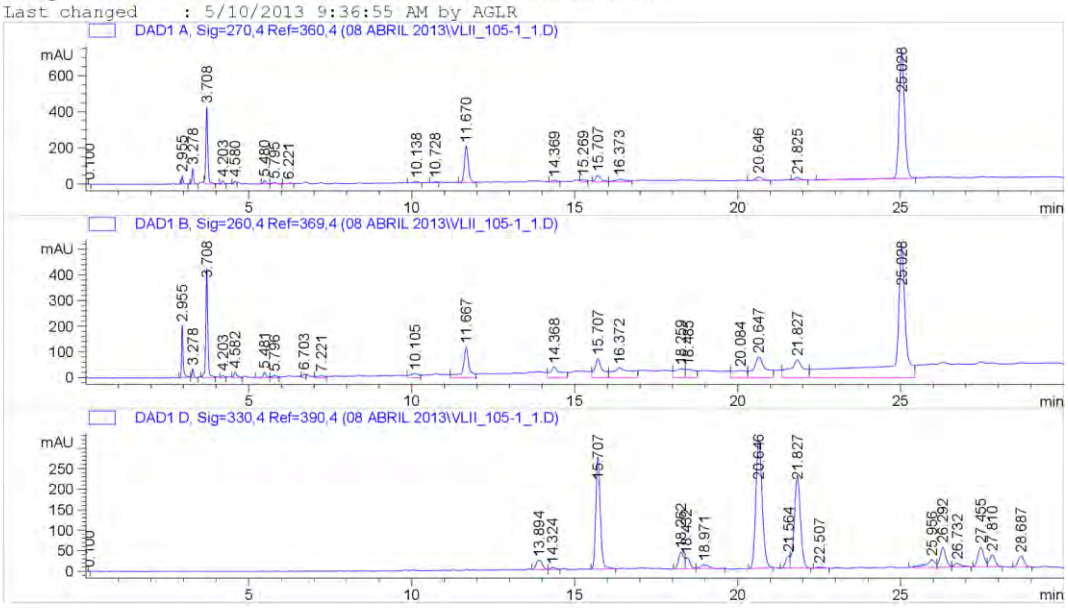
ANNEX 12. ^{1}H-NMR spectrum of an APU Classic coffee sample processed with ACD 12.01.



ANNEX 13. HPLC-DAD chromatogram of an instant coffee sample.

```
Data File D:\DATA\08 ABRIL 2013\VLII_105-1_1.D
Sample Name: VLII_105-1

======================================================================
Acq. Operator   : AGLR                          Seq. Line :   5
Acq. Instrument : Instrument 1                   Location : Vial 1
Injection Date  : 4/8/2013 11:54:00 AM               Inj :   1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII_CAFE1_020413.M
Last changed    : 4/8/2013 11:53:50 AM by AGLR
                  (modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 5/10/2013 9:36:55 AM by AGLR
```




```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   0.100 BB    0.0224    1.24086 8.84148e-1 8.700e-3
   2   2.955 BB    0.0492  149.25240   45.58075   1.0464
   3   3.278 BV    0.0512  290.56894   86.55850   2.0372
   4   3.708 VV    0.0650 1801.87158  428.62799  12.6330
   5   4.203 VV    0.0749   87.57989   17.64472   0.6140
   6   4.580 VV    0.0768   75.40541   14.97388   0.5287
   7   5.480 BV    0.0806   95.92579   18.18182   0.6725
   8   5.795 VB    0.1247   61.48545    6.98505   0.4311
   9   6.221 BV    0.1659   44.84925    3.56308   0.3144
  10  10.138 VV    0.1601  109.96507    9.56963   0.7710
  11  10.728 VV    0.1354   74.65417    8.18540   0.5234
  12  11.670 VV    0.1291 1723.01599  202.92496  12.0802
  13  14.369 BV    0.1750  129.63351   10.72688   0.9089
  14  15.269 VV    0.1687  141.24574   12.41366   0.9903
  15  15.707 VV    0.1724  409.85684   35.05677   2.8735
  16  16.373 VB    0.2882  295.69897   13.56917   2.0732
  17  20.646 VB    0.2113  279.57001   20.47859   1.9601

Instrument 1 6/15/2013 6:13:41 PM AGLR                    Page   1 of 2
```

Data File D:\DATA\08 ABRIL 2013\VLII_105-1_1.D
Sample Name: VLII_105-1

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
18	21.825	VB	0.1982	170.77531	13.08858	1.1973
19	25.028	BV	0.1839	8320.56738	693.71167	58.3361
Totals :				1.42632e4	1642.72523	

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.955	BV	0.0494	688.75226	204.10983	2.4169
2	3.278	VV	0.0572	135.05937	34.83580	0.4739
3	3.708	VV	0.0667	1854.46021	426.63943	6.5075
4	4.203	VV	0.0851	54.39255	9.18648	0.1909
5	4.582	VB	0.0977	143.76993	21.03473	0.5045
6	5.481	BV	0.1071	149.95398	20.08390	0.5262
7	5.796	VV	0.1442	93.44196	8.91868	0.3279
8	6.703	VV	0.0927	92.46006	14.44669	0.3244
9	7.221	VV	0.2267	128.72617	7.95863	0.4517
10	10.105	VV	0.2771	312.72253	16.20733	1.0974
11	11.667	BV	0.1834	1563.58496	118.92250	5.4867
12	14.368	VB	0.3016	951.88989	41.80557	3.3403
13	15.707	VV	0.2271	1215.11145	74.19037	4.2639
14	16.372	VB	0.4985	1554.55261	39.04449	5.4551
15	18.259	VV	0.2741	707.12543	35.24765	2.4814
16	18.485	VV	0.2895	681.03302	32.62783	2.3898
17	20.084	BV	0.4075	842.11523	27.78897	2.9551
18	20.647	VB	0.3493	2048.80176	80.40099	7.1894
19	21.827	BV	0.4046	2124.71631	69.13496	7.4558
20	25.028	VV	0.3468	1.31548e4	510.28085	46.1613
Totals :				2.84975e4	1792.86570	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

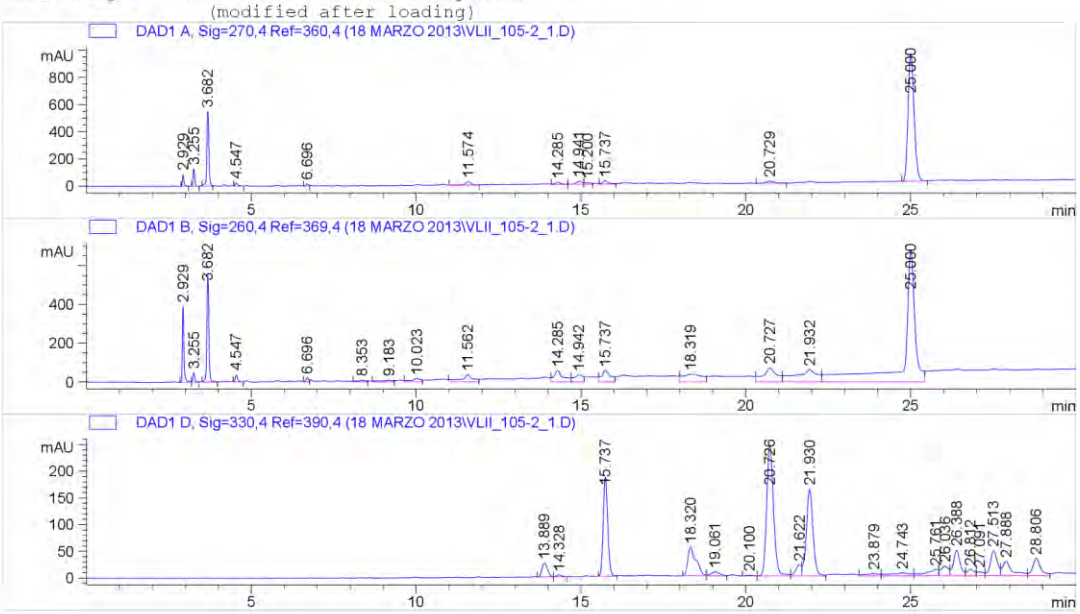
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.100	BB	0.0243	5.13568e-1	3.19394e-1	3.709e-3
2	13.894	VV	0.2174	308.31619	22.85117	2.2269
3	14.324	VV	0.1626	62.23375	5.27532	0.4495
4	15.707	BB	0.1476	2626.19214	273.42755	18.9682
5	18.262	BV	0.1737	479.68088	41.24984	3.4646
6	18.432	VB	0.1504	260.87161	26.60881	1.8842
7	18.971	BB	0.2288	172.56667	10.22981	1.2464
8	20.646	BB	0.2097	4225.18506	311.58359	30.5172
9	21.564	BV	0.1427	276.64728	30.11050	1.9981
10	21.827	VB	0.2013	2981.99121	226.27187	21.5380
11	22.507	BB	0.1747	45.12306	3.43576	0.3259
12	25.956	BV	0.2709	358.51505	18.11362	2.5894
13	26.292	VV	0.1855	593.17902	48.37995	4.2843
14	26.732	VB	0.2087	140.29810	9.67382	1.0133
15	27.455	BV	0.1966	612.06799	47.89571	4.4208
16	27.810	VB	0.1924	358.66293	28.28875	2.5905
17	28.687	BB	0.1968	343.22409	26.46526	2.4790
Totals :				1.38453e4	1130.18071	

*** End of Report ***

ANNEX 14. HPLC-DAD chromatogram of a Huayabamba coffee a sample.

```
Data File D:\DATA\18 MARZO 2013\VLII_105-2_1.D
Sample Name: VLII_105-2

==========================================================================
Acq. Operator   : AGLR                          Seq. Line :   5
Acq. Instrument : Instrument 1                   Location : Vial 2
Injection Date  : 4/8/2013 6:35:57 PM              Inj :   1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII_CAFE1_020413.M
Last changed    : 4/8/2013 6:35:47 PM by AGLR
                  (modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 6/15/2013 6:18:11 PM by AGLR
                  (modified after loading)
```




```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]      %
----|-------|----|-------|----------|----------|--------|
   1   2.929 BB    0.0490  287.78571   88.41312   1.6960
   2   3.255 BB    0.0535  453.21933  130.45580   2.6709
   3   3.682 VV    0.0649 2365.10229  552.54492  13.9380
   4   4.547 VB    0.0907  162.85689   26.14397   0.9597
   5   6.696 VV    0.0869   95.80285   16.70668   0.5646
   6  11.574 VV    0.1780  318.07092   25.77000   1.8745
   7  14.285 VV    0.1987  225.93846   16.61015   1.3315
   8  14.941 VV    0.1836  301.67426   24.16442   1.7778
   9  15.200 VV    0.1995  178.87364   13.42123   1.0541
  10  15.737 VV    0.1933  337.50250   25.35829   1.9890
  11  20.729 VB    0.2256  233.02161   16.03281   1.3732
  12  25.000 BB    0.1998 1.20088e4   935.15302  70.7706

Totals :                  1.69687e4  1870.77441


Instrument 1 6/15/2013 6:23:45 PM AGLR                    Page   1 of 2
```

Data File D:\DATA\18 MARZO 2013\VLII_105-2_1.D
Sample Name: VLII_105-2

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.929	BV	0.0491	1280.94360	392.60397	3.9943
2	3.255	VV	0.0569	188.92151	50.17788	0.5891
3	3.682	VB	0.0650	2348.47046	548.34540	7.3232
4	4.547	VV	0.0803	196.70596	36.84819	0.6134
5	6.696	VV	0.0913	134.67561	22.03929	0.4200
6	8.353	VV	0.2322	170.45998	9.69280	0.5315
7	9.183	VV	0.3701	282.74127	9.55855	0.8817
8	10.023	VV	0.2726	376.22144	18.55434	1.1732
9	11.562	VV	0.3225	954.81555	38.34663	2.9774
10	14.285	VB	0.2733	1161.83618	58.09340	3.6229
11	14.942	BV	0.2503	684.06403	37.94640	2.1331
12	15.737	VV	0.2503	1106.01294	60.20768	3.4488
13	18.319	VV	0.5216	1628.84680	39.61927	5.0792
14	20.727	VV	0.3895	2062.73364	71.72390	6.4322
15	21.932	VV	0.5886	2893.47583	62.44483	9.0226
16	25.000	VV	0.3345	1.65982e4	680.72443	51.7575
Totals :				3.20691e4	2136.92695	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

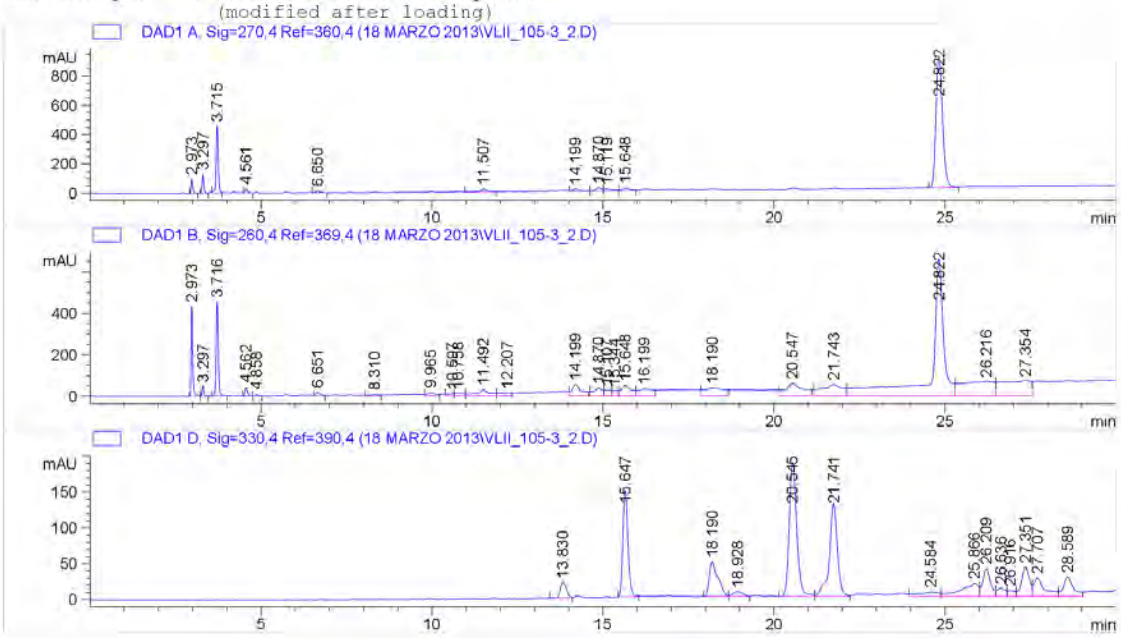
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.889	VV	0.1915	311.45905	26.05509	2.4008
2	14.328	VV	0.1610	41.71003	3.77152	0.3215
3	15.737	BB	0.1483	1785.20215	185.48859	13.7607
4	18.320	BV	0.2408	923.73395	53.66333	7.1203
5	19.061	VV	0.2993	139.58626	7.29247	1.0760
6	20.100	VV	0.2070	18.34484	1.34599	0.1414
7	20.726	VV	0.2210	3527.50806	243.54739	27.1907
8	21.622	VV	0.1799	245.64018	21.08189	1.8934
9	21.930	VB	0.2113	2265.83521	161.92746	17.4655
10	23.879	VV	0.3839	122.74274	4.31442	0.9461
11	24.743	VV	0.5108	230.35500	5.75703	1.7756
12	25.761	VV	0.3245	305.17310	12.17311	2.3523
13	26.036	VV	0.2266	289.38501	18.08885	2.2306
14	26.388	VV	0.2091	678.55511	47.95916	5.2304
15	26.812	VV	0.2172	180.01407	11.85083	1.3876
16	27.091	VV	0.2059	101.52995	7.13873	0.7826
17	27.513	VV	0.2262	702.17542	47.01759	5.4125
18	27.888	VV	0.2843	547.37018	27.02520	4.2192
19	28.806	VV	0.2529	556.91107	32.33331	4.2928
Totals :				1.29732e4	917.83196	

*** End of Report ***

ANNEX 15. HPLC-DAD chromatogram of a Huayabamba coffee b sample.

```
Data File D:\DATA\18 MARZO 2013\VLII_105-3_2.D
Sample Name: VLII_105-3

==========================================================================
Acq. Operator    : AGLR                          Seq. Line :  21
Acq. Instrument : Instrument 1                    Location : Vial 3
Injection Date  : 4/9/2013 3:06:46 AM                 Inj :  1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII CAFE1_020413.M
Last changed    : 4/9/2013 3:06:35 AM by AGLR
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 6/15/2013 6:18:11 PM by AGLR
                  (modified after loading)
```




```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.973 BB    0.0492  318.97769   97.34015   1.9503
   2   3.297 BB    0.0528  444.88229  127.21387   2.7201
   3   3.715 VV    0.0655 1996.88440  461.21387  12.2093
   4   4.561 VV    0.0849  172.63002   29.67442   1.0555
   5   6.650 VB    0.1153  125.33141   15.33751   0.7663
   6  11.507 VV    0.1942  247.63232   18.49772   1.5141
   7  14.199 VV    0.2096  229.43610   15.78416   1.4028
   8  14.870 VV    0.1773  283.50226   23.74544   1.7334
   9  15.119 VV    0.2453  248.11324   13.96123   1.5170
  10  15.648 VV    0.2041  299.70178   20.80108   1.8324
  11  24.822 BB    0.2054 1.19884e4   900.05756  73.2989

Totals :                  1.63555e4  1723.62700
```

Data File D:\DATA\18 MARZO 2013\VLII_105-3_2.D
Sample Name: VLII_105-3

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.973	BV	0.0495	1434.63904	435.10492	3.2849
2	3.297	VV	0.0573	192.17267	49.48302	0.4400
3	3.716	VB	0.0660	2000.04004	457.90411	4.5794
4	4.562	VV	0.0780	217.52205	42.26168	0.4981
5	4.858	VB	0.0831	48.48909	8.96404	0.1110
6	6.651	VV	0.1281	186.41470	20.12148	0.4268
7	8.310	VB	0.2387	163.82457	9.06953	0.3751
8	9.965	BB	0.2359	311.84140	18.01811	0.7140
9	10.597	BV	0.1823	170.68677	12.57862	0.3908
10	10.758	VV	0.2414	251.68700	14.15577	0.5763
11	11.492	VB	0.3639	887.17047	31.37031	2.0313
12	12.207	BV	0.3411	437.67731	16.73788	1.0021
13	14.199	VB	0.2700	1123.09717	56.95658	2.5715
14	14.870	BV	0.2551	708.74194	38.09322	1.6228
15	15.107	VV	0.1933	388.48111	29.17476	0.8895
16	15.344	VV	0.1594	302.00644	26.80840	0.6915
17	15.648	VV	0.2746	1085.34460	53.50863	2.4851
18	16.199	VV	0.3916	1085.09363	35.59721	2.4845
19	18.190	VV	0.5232	1638.32703	39.72115	3.7512
20	20.547	VB	0.4849	2300.11353	62.56226	5.2665
21	21.743	BV	0.5504	2454.27319	57.45199	5.6195
22	24.822	VV	0.3523	1.71131e4	660.36102	39.1834
23	26.216	VV	0.8086	4687.26855	71.70997	10.7323
24	27.354	VV	0.7531	4486.39209	74.86845	10.2724
Totals :				4.36744e4	2322.58310	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.830	BV	0.2075	282.82635	21.77711	2.4832
2	15.647	BB	0.1562	1531.91516	148.66016	13.4502
3	18.190	BV	0.2490	847.17865	48.20213	7.4382
4	18.928	VV	0.3028	129.27467	6.53391	1.1350
5	20.545	VV	0.2346	2859.69531	186.76117	25.1082
6	21.741	VV	0.2453	2141.19189	129.22592	18.7997
7	24.584	VV	0.5421	247.79091	5.94720	2.1756
8	25.866	VV	0.4478	639.22522	18.28740	5.6124
9	26.209	VV	0.2308	607.23047	38.76247	5.3315
10	26.636	VV	0.2343	188.27399	11.54491	1.6530
11	26.916	VV	0.2171	121.53082	8.19243	1.0670
12	27.351	VV	0.2403	663.15771	41.09184	5.8225
13	27.707	VV	0.3127	576.86884	25.42680	5.0649
14	28.589	VV	0.2950	553.34290	27.01375	4.8584
Totals :				1.13895e4	717.42720	

*** End of Report ***

ANNEX 16. HPLC-DAD chromatogram of an APU Espresso coffee sample.

```
Data File D:\DATA\09 ABRIL 2013\VLII_105-4_2.D
Sample Name: VLII_105-4

======================================================================
Acq. Operator   : AGLR                          Seq. Line :   1
Acq. Instrument : Instrument 1                   Location : Vial 4
Injection Date  : 4/9/2013 11:40:13 AM              Inj :   1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII_CAFE1_020413.M
Last changed    : 4/9/2013 11:40:02 AM by AGLR
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 5/10/2013 9:36:55 AM by AGLR
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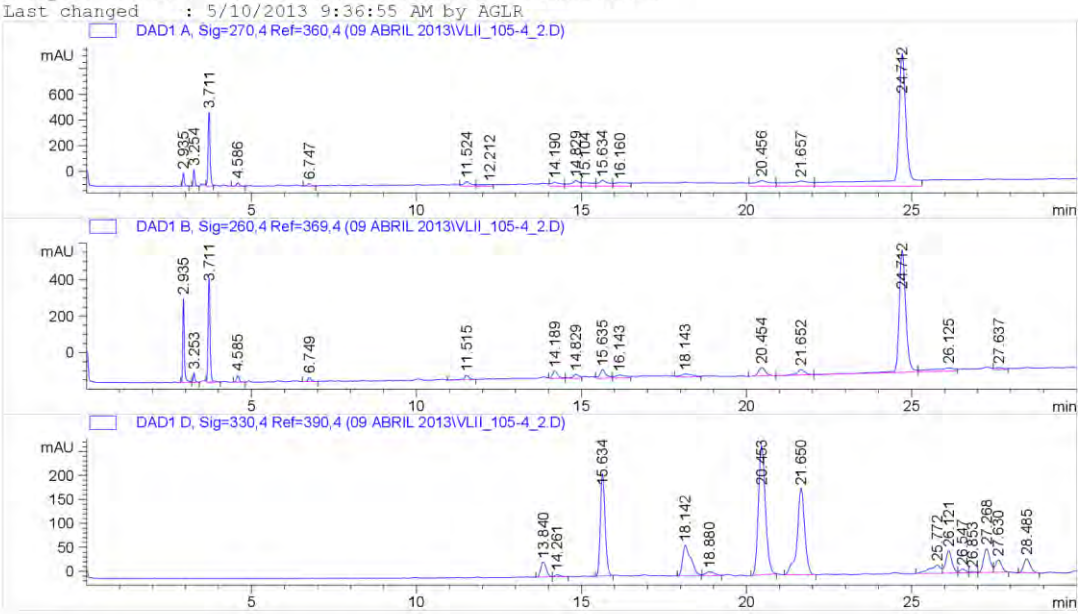



```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]      %
----|-------|----|-------|----------|----------|--------|
   1   2.935 BB    0.0491  337.94724  103.43000   1.0268
   2   3.254 BV    0.0526  455.24014  130.97636   1.3832
   3   3.711 VV    0.0645 2445.49414  576.11725   7.4302
   4   4.586 BV    0.1116  207.46228   26.70214   0.6303
   5   6.747 BB    0.1324  195.08578   21.20020   0.5927
   6  11.524 BV    0.2241  595.84869   36.96142   1.8104
   7  12.212 VV    0.3699  413.10709   14.60997   1.2552
   8  14.190 VV    0.2759  646.98987   31.99215   1.9658
   9  14.829 VV    0.2584  834.78650   44.18629   2.5363
  10  15.104 VV    0.3087  683.92419   29.69414   2.0780
  11  15.634 VV    0.2717  929.19531   46.38950   2.8232
  12  16.160 VV    0.3886  886.90021   29.18212   2.6947
  13  20.456 VV    0.4665 1541.06104   43.58611   4.6822
  14  21.657 VV    0.6962 2201.02686   39.73986   6.6874
  15  24.712 VB    0.2819 2.05389e4  1033.17090  62.4037

Totals :                  3.29130e4  2207.93840

Instrument 1 6/15/2013 6:17:24 PM AGLR                    Page   1 of 2
```

Data File D:\DATA\09 ABRIL 2013\VLII_105-4_2.D
Sample Name: VLII_105-4

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.935	BV	0.0488	1491.22424	460.23480	7.9113
2	3.253	VV	0.0562	192.22705	50.72707	1.0198
3	3.711	VV	0.0656	2477.53906	571.16089	13.1439
4	4.585	BV	0.0864	215.05281	37.22639	1.1409
5	6.749	VB	0.1076	160.40732	22.92016	0.8510
6	11.515	VV	0.1793	319.76584	25.66966	1.6964
7	14.189	VV	0.1841	518.53406	41.98755	2.7509
8	14.829	VV	0.2066	326.13034	22.84101	1.7302
9	15.635	VV	0.1904	600.33618	45.97009	3.1849
10	16.143	VV	0.3111	369.73318	15.79306	1.9615
11	18.143	VV	0.3570	478.49405	17.72175	2.5385
12	20.454	VV	0.2294	674.10767	45.34713	3.5763
13	21.652	VV	0.2382	479.20718	30.02723	2.5423
14	24.712	VV	0.2156	9428.72559	672.58179	50.0215
15	26.125	VV	0.6130	876.83679	18.10683	4.6518
16	27.637	VV	0.2998	241.02019	11.16916	1.2787
Totals :				1.88493e4	2089.48456	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

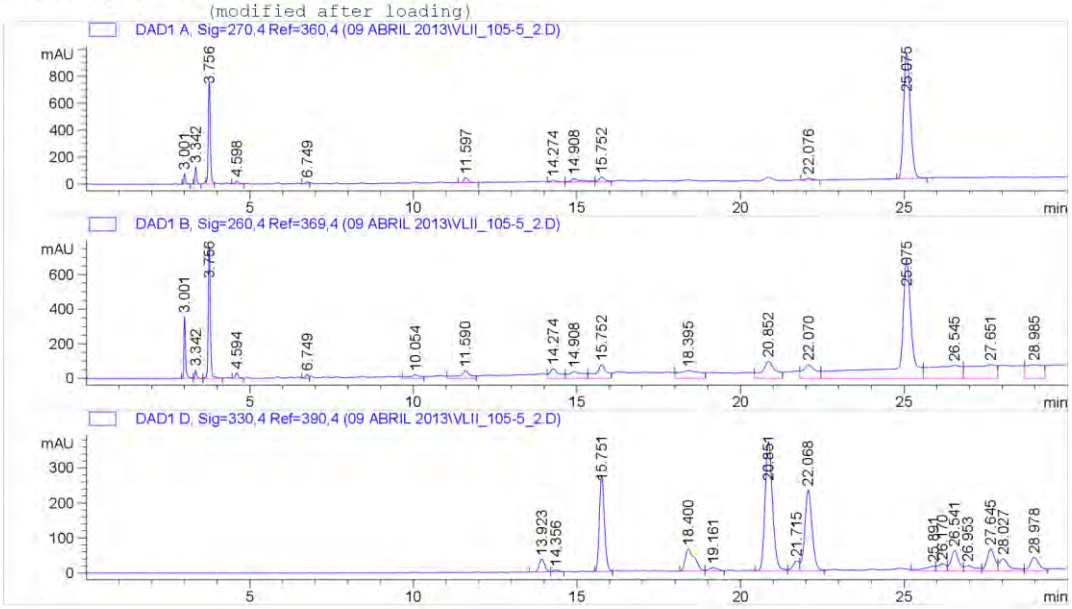
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.840	BV	0.1869	367.19891	31.18213	2.7631
2	14.261	VB	0.1608	49.16617	4.43711	0.3700
3	15.634	BB	0.1542	2128.16699	212.82550	16.0141
4	18.142	BB	0.2453	1114.17773	63.47647	8.3840
5	18.880	BV	0.2503	161.75597	8.30102	1.2172
6	20.453	BB	0.2288	4001.59326	269.43417	30.1114
7	21.650	BB	0.2363	2882.56812	181.00891	21.6909
8	25.772	BV	0.3060	405.23438	17.31720	3.0493
9	26.121	VV	0.2008	623.45966	46.83813	4.6914
10	26.547	VV	0.1781	100.63367	8.09352	0.7573
11	26.853	VV	0.1462	29.05849	2.50768	0.2187
12	27.268	VV	0.2111	669.61475	49.25798	5.0387
13	27.630	VB	0.2022	348.83676	25.73136	2.6249
14	28.485	BB	0.2134	407.84940	29.38714	3.0690
Totals :				1.32893e4	949.79834	

*** End of Report ***

ANNEX 17. HPLC-DAD chromatogram of an APU Gourmet coffee sample.

```
Data File D:\DATA\09 ABRIL 2013\VLII_105-5_2.D
Sample Name: VLII_105-5

======================================================================
Acq. Operator   : AGLR                          Seq. Line :  13
Acq. Instrument : Instrument 1                   Location : Vial 5
Injection Date  : 4/9/2013 6:03:00 PM               Inj :   1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII_CAFE1_020413.M
Last changed    : 4/9/2013 6:02:49 PM by AGLR
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 6/15/2013 6:18:11 PM by AGLR
                  (modified after loading)
```




```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   3.001 BB    0.0530  279.96701   79.74111   1.4147
   2   3.342 BB    0.0571  480.90005  127.24385   2.4301
   3   3.756 VV    0.0675 3373.98242  765.03198  17.0492
   4   4.598 VV    0.1058  167.27530   22.73273   0.8453
   5   6.749 VV    0.0975  108.87657   16.60319   0.5502
   6  11.597 BB    0.1609  379.27371   36.00114   1.9165
   7  14.274 VV    0.2126  224.99586   15.38748   1.1369
   8  14.908 VV    0.3027  566.24463   25.34122   2.8613
   9  15.752 VV    0.1962  485.92221   36.29354   2.4554
  10  22.076 VV    0.2342  227.35063   14.88586   1.1488
  11  25.075 BB    0.2243 1.34949e4   935.60236  68.1916

Totals :                  1.97897e4  2074.86446


Instrument 1 6/15/2013 6:19:28 PM AGLR                    Page   1 of 2
```

Data File D:\DATA\09 ABRIL 2013\VLII_105-5_2.D
Sample Name: VLII_105-5

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.001	BV	0.0535	1270.28809	357.08331	2.5377
2	3.342	VV	0.0642	217.66170	49.63888	0.4348
3	3.756	VV	0.0701	3460.74365	759.97638	6.9138
4	4.594	VV	0.0984	222.15028	33.04148	0.4438
5	6.749	VV	0.1107	186.19037	24.22701	0.3720
6	10.054	VV	0.3244	545.13434	22.20826	1.0891
7	11.590	VV	0.3213	1148.62537	46.96260	2.2947
8	14.274	VV	0.2797	1178.32813	57.82445	2.3540
9	14.908	VV	0.4006	1299.15930	42.03496	2.5954
10	15.752	VV	0.3043	1836.00098	81.04060	3.6679
11	18.395	VV	0.5870	2159.99805	46.58127	4.3152
12	20.852	VV	0.3938	2836.20532	99.12901	5.6661
13	22.070	VV	0.3753	2197.09033	80.28186	4.3893
14	25.075	VV	0.3761	1.88752e4	687.98444	37.7084
15	26.545	VV	0.8308	5104.73340	76.47031	10.1981
16	27.651	VV	0.7255	4634.46582	80.75533	9.2586
17	28.985	BV	0.4853	2883.79248	79.86311	5.7612
Totals :				5.00558e4	2625.10323	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

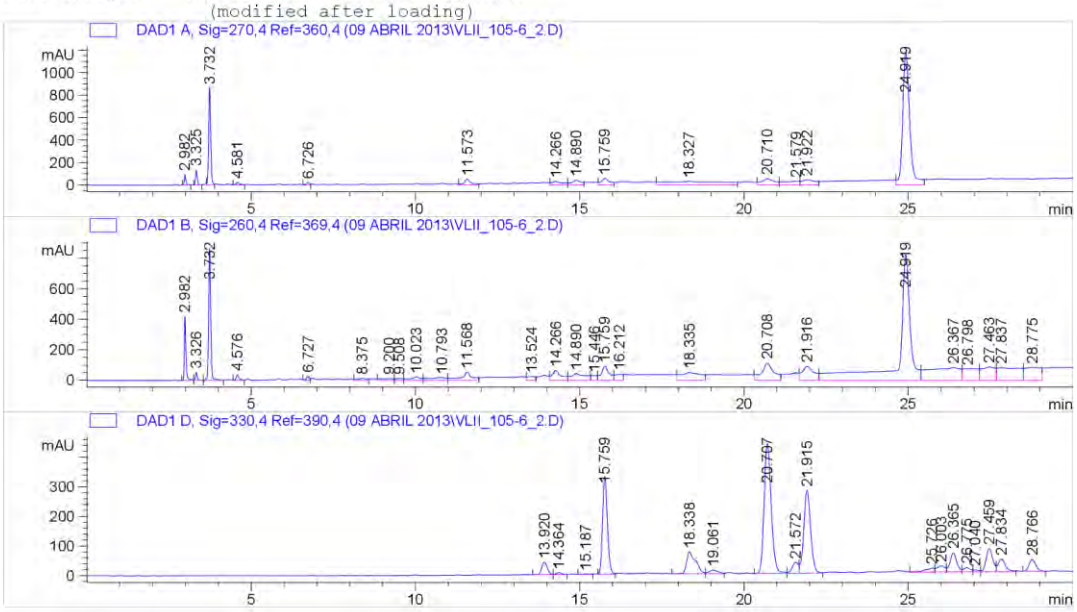
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.923	BV	0.1965	451.73599	36.46301	2.3196
2	14.356	VV	0.1751	65.79117	5.51642	0.3378
3	15.751	BV	0.1653	2951.32227	274.75482	15.1545
4	18.400	BV	0.2777	1258.05823	62.79927	6.4599
5	19.161	VV	0.2880	185.79959	9.85291	0.9540
6	20.851	VV	0.2463	5909.74463	370.06036	30.3454
7	21.715	VV	0.2013	388.96332	28.84419	1.9973
8	22.068	VV	0.2396	3648.60034	231.89314	18.7349
9	25.891	VV	0.3379	360.30090	13.92137	1.8501
10	26.170	VV	0.2589	367.97299	20.74839	1.8895
11	26.541	VV	0.2435	959.16766	58.44506	4.9251
12	26.953	VV	0.3167	363.22058	15.54096	1.8651
13	27.645	VV	0.2494	1038.73462	62.67113	5.3337
14	28.027	VV	0.3014	757.58240	35.43314	3.8900
15	28.978	VV	0.2936	767.91986	39.01458	3.9431
Totals :				1.94749e4	1265.95879	

*** End of Report ***

ANNEX 18. HPLC-DAD chromatogram of an APU Classic coffee sample.

```
Data File D:\DATA\09 ABRIL 2013\VLII_105-6_2.D
Sample Name: VLII_105-6

======================================================================
Acq. Operator   : AGLR                          Seq. Line :  25
Acq. Instrument : Instrument 1                   Location : Vial 6
Injection Date  : 4/10/2013 12:25:08 AM             Inj :  1
                                                 Inj Volume : 20 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 10 µl
Acq. Method     : C:\CHEM32\1\METHODS\VLII_CAFE1_020413.M
Last changed    : 4/10/2013 12:24:57 AM by AGLR
Analysis Method : C:\CHEM32\1\METHODS\LAVADO FENYLHEXYLO.M
Last changed    : 6/15/2013 6:18:11 PM by AGLR
                  (modified after loading)
```




```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=270,4 Ref=360,4

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.982 BB    0.0513  316.77484   91.81055   0.9145
   2   3.325 BB    0.0572  507.78738  133.94655   1.4659
   3   3.732 VV    0.0666 3772.50732  870.57196  10.8907
   4   4.581 VV    0.1242  212.13698   24.68814   0.6124
   5   6.726 VV    0.1068  161.64734   21.96729   0.4667
   6  11.573 BV    0.2197  862.79840   54.20218   2.4908
   7  14.266 VV    0.3105  774.84497   33.41885   2.2369
   8  14.890 VV    0.2673  893.16907   45.43620   2.5784
   9  15.759 VV    0.2457 1087.18921   61.06400   3.1386
  10  18.327 VV    1.4586 4239.23145   34.91314  12.2380
  11  20.710 VV    0.3759 1588.37830   57.93498   4.5854
  12  21.579 VV    0.4452 1222.53442   36.08657   3.5293
  13  21.922 VV    0.3779 1356.57690   49.16004   3.9162
  14  24.919 BB    0.2292 1.76442e4  1174.90881  50.9363

Totals :                  3.46398e4  2690.10926
```

Data File D:\DATA\09 ABRIL 2013\VLII_105-6_2.D
Sample Name: VLII_105-6

Signal 2: DAD1 B, Sig=260,4 Ref=369,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.982	BV	0.0510	1437.89392	419.39804	2.3560
2	3.326	VV	0.0646	225.83221	52.02773	0.3700
3	3.732	VB	0.0681	3864.12622	865.92731	6.3314
4	4.576	VV	0.0987	248.24522	36.78766	0.4068
5	6.727	VV	0.1061	196.70392	26.97507	0.3223
6	8.375	VV	0.2500	274.28641	14.68190	0.4494
7	9.200	BV	0.3336	336.69379	12.98222	0.5517
8	9.508	VV	0.2128	206.73776	13.48654	0.3387
9	10.023	VV	0.3085	540.06940	23.29663	0.8849
10	10.793	VV	0.4510	704.57397	19.52347	1.1545
11	11.568	VV	0.3087	1290.21375	54.78844	2.1140
12	13.524	VV	0.2333	448.44534	25.47846	0.7348
13	14.266	VV	0.2733	1287.50964	64.39731	2.1096
14	14.890	VV	0.3957	1404.44556	46.06123	2.3012
15	15.446	VV	0.1827	421.93628	32.67396	0.6914
16	15.759	VV	0.2378	1581.00610	93.24889	2.5905
17	16.212	VV	0.2212	671.88074	42.32521	1.1009
18	18.335	VV	0.5428	2244.17505	52.27834	3.6771
19	20.708	VV	0.3666	3021.26538	113.49943	4.9504
20	21.916	VV	0.3486	2319.49585	91.84118	3.8005
21	24.919	VV	0.3471	2.08721e4	825.14545	34.1993
22	26.367	VV	0.8380	5700.57080	84.18922	9.3405
23	26.798	VB	0.4037	2394.45435	76.80370	3.9234
24	27.463	BV	0.3879	2541.76587	88.81373	4.1647
25	27.837	VB	0.6081	3992.26074	83.75943	6.5414
26	28.775	BV	0.4373	2804.01953	85.78487	4.5944
Totals :				6.10307e4	3346.17540	

Signal 3: DAD1 D, Sig=330,4 Ref=390,4

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.920	BV	0.1871	499.74399	41.92063	2.3743
2	14.364	VV	0.1794	80.26141	6.71951	0.3813
3	15.187	VB	0.2353	29.01979	1.66557	0.1379
4	15.759	BV	0.1600	3385.47803	329.08249	16.0842
5	18.338	VV	0.2701	1431.18030	73.84863	6.7994
6	19.061	VV	0.2930	234.35811	12.15154	1.1134
7	20.707	VV	0.2320	6607.38135	437.93741	31.3912
8	21.572	VV	0.1939	469.23361	36.51356	2.2293
9	21.915	VV	0.2214	4059.05957	279.62518	19.2843
10	25.726	VV	0.2807	295.93698	13.88470	1.4060
11	26.003	VV	0.2388	359.10733	21.51006	1.7061
12	26.365	VV	0.2096	921.99219	64.96165	4.3803
13	26.775	VV	0.2138	209.33844	14.38718	0.9946
14	27.040	VV	0.1780	76.16720	6.43909	0.3619
15	27.459	VV	0.2195	1122.08252	78.16434	5.3309
16	27.834	VB	0.2311	666.61096	42.46855	3.1670
17	28.766	BV	0.2347	601.54102	40.17242	2.8579
Totals :				2.10485e4	1501.45250	

*** End of Report ***