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Analysis of Antiphysiological Components of Coffee Pulp

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Coffee is a major cash crop in several countries all over the world. After removal of the two seeds present in each berry, the fleshy part of the fruit, or coffee pulp, is discarded. Coffee pulp, which is presently produced around 14 million tons worldwide, is a by-product of the processing of coffee beans. Much of this pulp is currently dumped in watercourses or on roadsides where it causes serious pollution. For obvious economic and environmental reasons major efforts are underway to upgrade coffee pulp. Attempts have been made to utilize coffee pulp as animal feed, without much success. Phenolic compounds (tannins) and caffeine show anti-nutritional effects of this otherwise-rich by-product (Roussos et al 2000). The analysis of polyphenolic constituents revealed major constituents such as flavan-3-ols or chlorogenic acid. Caffeine levels were also measured. Endogenous fungal strains were isolated from coffee pulp and characterized. Filamentous and edible mushroom, able to degrade both caffeine and specific polyphenols were characterized. The biodegradation pathways of caffeine (Hakil et al 1998) and specific flavan-3-ol constituents (catechin) were elucidated. Some enzymes involved in the degradation pathways were identified and characterized. Even though the environmental problem of coffee pulp has not been solved, potential uses could soon transform this by-product into a value added co-product through biotechnological processes.

Keywords: coffee, coffee pulp, microorganisms, biotechnology, caffeine, tannins, degradation, fungus, *Aspergillus, Penicillium.*

1. INTRODUCTION

Coffee is one of the most important tropical cultures in the world, not only for the 56 countries of the "developing" World which cultivate it but also for the United States, Europe and Japan which together consume nearly 80% of the produced coffee. The coffee cherry is the fruit of the coffee tree, belonging to the Rubiace family, which the genus *Coffea* belongs to (Coste 1989). There are approximately sixty species within the *Coffea* genus. The original coffee tree is said to have come from Ethiopian mountains in Africa. The plant requires a hot and wet climate typically tropical (temperatures ranging between 17 and 23°C and 1500 to 1800 mm of precipitation per annum). Harvest of the fruit depends on the area, the latitude and the altitude which can vary from sea level (*C. canephora*) to nearly 2000 meters (*C. arabica*). Flowering of the coffee plant varies according to the area, the latitude and the species. Approximately 28 weeks go by between flowering and fructification; fruit maturation is between 5 to 6 months. The fruit of the coffee tree has the same appearance as that of a cherry, therefore being often called «coffee cherry». Fruit grow along the

branches (Wilbaux 1956). They are ovoid in form and the color varies from yellow to red according to the variety and location. The red fruit are generally considered of better quality. The size varies according to variety, on average: 10 mm in length, 6–7 mm in width. In most countries, coffee is generally hand-picked, as fruit mature sequentially and quality is highly linked to maturity. In Brazil, cherries are gathered branch by branch ("strip picking") or by machine. The percentage of immature berries is much higher, which results in a lower quality of the drink (Wilbaux, 1956; Coste, 1989). Ripe fruit are processed immediately, on or near the site of production. The fruit undergo a certain number of steps, in order to remove the seeds from their fleshy part (pulp and mucilage). Seeds are then dried before shipping. There are two techniques used, in order to obtain the commercial grain (Figure 1). The first is a method known as the "wet process" carried out in most of the world except Brazil. The berry is washed in water and then the fleshy part (pulp) and mucilage are removed and the seeds processed and dried. The "dry process" is used mainly in Brazil and on coffee berries of the *Robusta* variety. The fruit is dried and then the seeds are removed by breaking open the outer hull. In both cases, the seed ends up as the universal drink consumed all over the world. However, what is actually consumed represents only 6% of the original product, the coffee berry, leaving 94% as waste, mainly by-products from the seed-obtaining process (Zuluaga 1981).

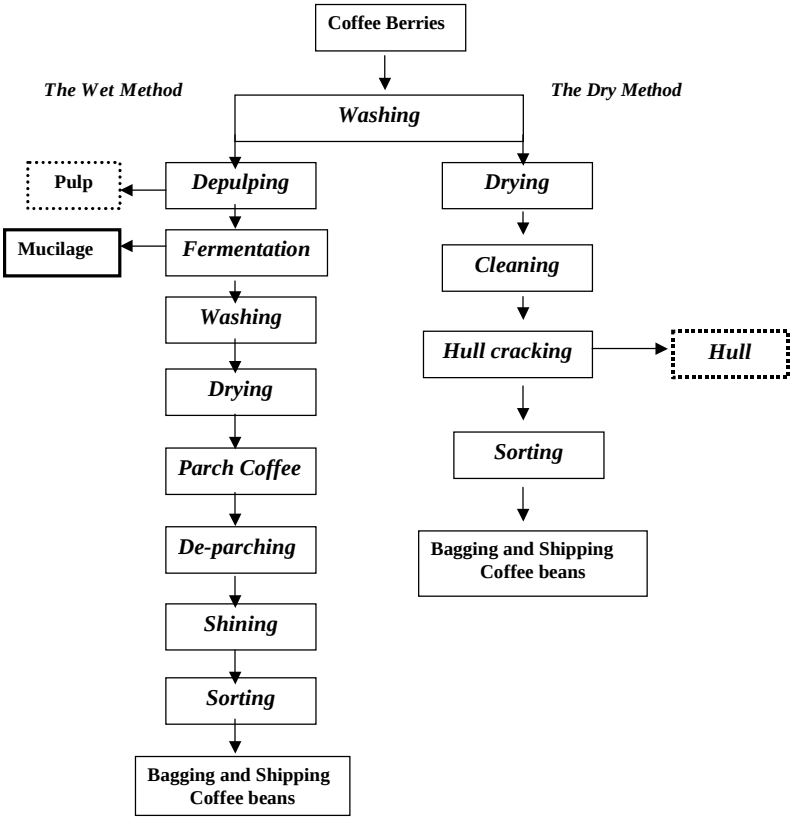


Figure 1. Steps involved in coffee production through the wet method and the dry method.

Mexico, which occupies fourth place in world coffee production, annually produces approximately 500,000 tons of coffee beans, primarily by using the "wet process" over a five-month period of the coffee season. The production of each ton of coffee grain also generates half a ton of coffee pulp. Although rich in proteins, reducing sugars and vitamins, coffee pulp is currently greatly underutilized. In spite of a potentially high food value, the pulp is mostly discarded as a waste, thus polluting the lakes and rivers bordering the sites of coffee production. For obvious ecological and economic reasons, the valorization of coffee pulp has become one of the priorities of producer countries. Considering the huge volume of this agro-industrial waste, its rapid decomposition, its potential source as feedstuff, fresh pulp first needed to be stabilized. This was achieved by using lactic bacteria in a silage technique. Coffee pulp could be ensiled right through the coffee season (Roussos et al 2000). The use of coffee pulp as cattle feed was suggested and tested (Bressani et al 1980) without success, in spite of the high content of carbohydrate and protein. The presence in the pulp of anti-nutritional compounds such as caffeine (1%) and the tannins (4–8%), were advanced as the reason of this failure. These substances could, directly or indirectly, have a toxic or antiphysiological effect. Before being able to attempt to put a relation of cause and effect between the presence of tannins in coffee pulp and its potential antiphysiological effect, there was an initial need to know the composition of tannins present in pulp of coffee. It should be noted that the term «tannin», often employed very freely, groups many phenolic compounds with often very different structures (Waterman et al 1994). These compounds are water-soluble with the ability and characteristic of being able to complex and precipitate proteins and other macromolecules (Porter 1989). Tannins are divided into two great groups, namely condensed tannins, also known as proanthocyanidins, (Hemingway et al 1989) and hydrolysable tannins (Clifford *et a.* 2000, Okuda et al 1993). Hydrolysable tannins are composed of a sugar core (in general glucose) esterified by gallic acid. Each gallic acid can in turn be esterified, forming more complex molecules, like the tannic acid. The second class or category of tannins is known as condensed tannins. Some condensed tannins, also known as proanthocyanidins have a basic unit of the flavan-3-ol type, linked in C4-C8 and less frequently in C4–C6 (Czochanska et al 1980). The basic unit is often catechin or epicatechin (Figure 2). Proanthocyanidins account for 1 to 2.7% of fresh coffee pulp.

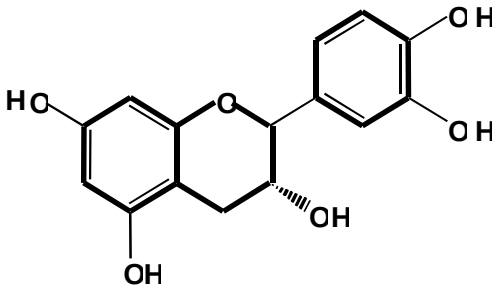


Figure 2. Structure of Epicatechin.

Tannins accumulate in leaves, roots, bark and fruits. Plants with high tannin content have an unquestionable advantage in evolutionary terms. They deter herbivores (Feeny 1970) and confer a resistance towards certain pathogenic micro-organisms (Brownlee et al 1990). Both hydrolysable and condensed tannins are present in fruits, which we consume on a daily basis, such as apples, almonds,

grapes or strawberries (Santos-Buelga et al 2000). Tannins have been shown to have beneficial properties with regards to medicine and health (Rice-Evans et al 1997). Hydrolysable tannins have been shown to induce apoptosis (programmed cellular death) of cancerous cells (Min-Hsiung et al 1999) or to inhibit the proliferation of cancerous cells (Lea et al 1993, Stoner et al 1995). Condensed tannins are effective as antioxidants (Gutteridge et al 1994) and some, such as (+)-catechin, can reduce the cholesterol levels in mice (Bursill et al 2000). However, it was shown that the polymerized forms of these catechins, those known to interact with proteins, reducing the nutritive potential, are not degraded when passing through the intestinal tracts of chicken (Jimenez-Ramsey et al 1994) or of goats (Terrill et al 1994). Finally, when calves were fed a 15% daily ration of coffee pulp, growth was stunted (Bressani et al 1980). Were condensed tannins solely responsible? The answer could come from the obtaining of a pulp with degraded tannins. However, before attempting to degrade coffee pulp tannins, it is essential to know in detail the types of tannins present in coffee pulp.

2. BIODEGRADATION OF TANNINS BY FILAMENTOUS FUNGI

Our initial work was geared towards the understanding of the structures of condensed tannins present in coffee pulp. Work on the composition of coffee pulp tannins is scarce and the studies are vague, mainly due to the lack of standards (for condensed tannins) and due to impossibility of measuring with precision hydrolysable tannins in coffee pulp. Coffee pulp from arabica cherries contain between 2-6% tannins, but the value largely could be underestimated (González De Colmenares et al 1994, 1998). Moreover, analytical methods are often nonspecific resulting in a set of sometimes-contradictory data like the presence or the absence of hydrolysable tannin in coffee pulp. Recently, the use of the thiolysis method made it possible to characterize and quantify condensed tannins in plants and fruits such as apple, pear or grape (Guyot et al 2001). This method consists in heating proanthocyanidins in the presence of HCl and a nucleophile such as toluene-alpha-thiol. The terminal units are released by acid hydrolysis and the extension units in the chain give benzyl-thio-ether derivatives. Thiolysis can also be used to know the degree of polymerization of these proanthocyanidins after separation of thiolysis products on HPLC. The above methodologies were applied to the analysis of the phenolic compounds of coffee pulp (Ramirez Coronel 2004).

Analyses were carried out on fresh pulp and pulp dried in the sun for 72 hours (called oxidized pulp). The polyphenols present were analyzed after direct thiolysis of freeze-dried pulp and also after successive extractions with methanol and acetone. Table 1 illustrates the various classes of identified compounds. Four groups of phenolic compounds were identified in coffee pulp. All the phenolic compounds present in fresh coffee pulp were also present in oxidized coffee pulp. The first group above, the flavan-3-ols represent more than 60% of the phenolic compounds obtained starting from a methanolic extract of fresh coffee pulp. HPLC analysis of samples subjected to thiolysis allows the determination not only of the nature but also of the proportion of constitutive units of proanthocyanidins by making the distinction between the final unit and the units of extension, that separate out with different retention times. Catechin was present only as the terminal unit. An example of separation by HPLC of an extract of fresh coffee pulp is shown in Figure 3. Several peaks of elution, measured according to their increasing retention times, as well as their absorbance spectrum were observed. Peaks corresponded to monomers to hexamers of proanthocyanidins. Caffeine was also present in the extract but elutes before the oligomers. In order to confirm the presence of these oligomers, the same sample was analyzed by mass spectrometry (MALDI-TOF-MS). The results are presented in

Table 1. Molecules identified by HPLC following two treatments of coffee pulp, before thiolysis (Bf) and after thiolysis (Af). I, corresponds to the identified molecules and X corresponds to non-detected molecules; SNA: standards not available. The molecules were identified (I) by their retention time (tR) on HPLC and by comparison of the spectra with those of standards

Group	Molecule	t_R	Lambda max	Fresh Pulp		Oxidized Pulp	
				Bf	Af	Bf	Af
Flavan-3-ol	Catechin	15.1	279.3	X	I	X	I
	Epicatechin	19.5	279.3	I	I	I	I
	Epicatechin-SR	40.71	279.3	X	I	X	I
	Procyanidin B2	17.35	279.3	I	X	I	X
Hydroxycinnamic Acids	Chlorogenic Acid	16.45	325.5	I	I	I	I
	p-Coumaric Acid	20.6	311.3	I	I	I	I
Flavonols	SNA		254.5–354.1	I	I	I	I
Anthocyanidins	SNA		280.6–516.8	I	I	I	I
Alcaloïd	Caffeine	17.8	273.4	I	I	I	I

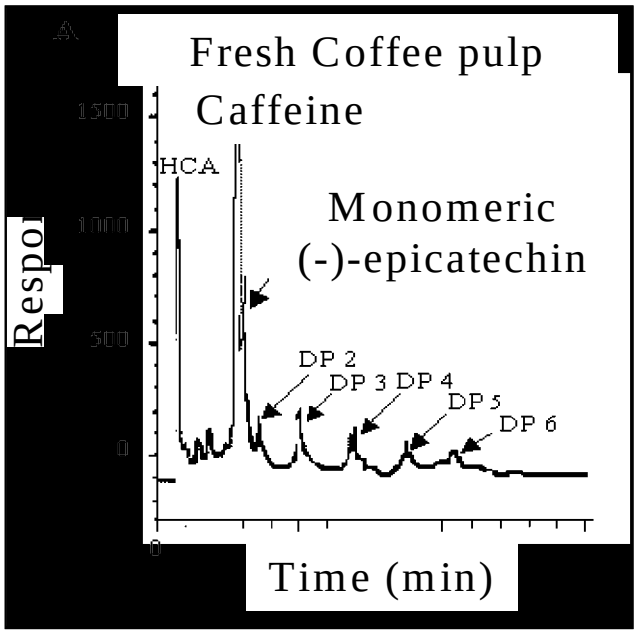
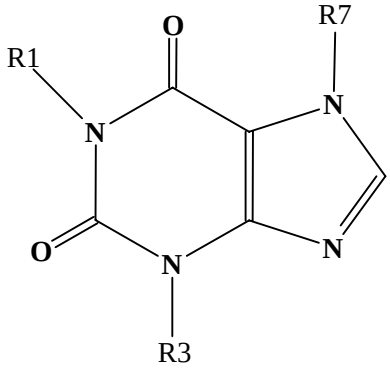


Figure 3. Separation by HPLC of an extract of fresh coffee pulp.

Figure 4. The molecular weights were in agreement with the previous data indicating that proanthocyanidins from monomer to at least hexamers were present in coffee pulp and were separated by chromatographic methods.



Purines	R1	R3	R7
1,3,7-Trimethylxanthine (caffeine)	CH_3	CH_3	CH_3
1,3-Dimethylxanthine (theophylline)	CH_3	CH_3	H
1,7-Dimethylxanthine (paraxanthine)	CH_3	H	CH_3
3,7-Dimethylxanthine (theobromine)	H	CH_3	CH_3
1-Methylxanthine	CH_3	H	H
3-Methylxanthine	H	CH_3	H
7-Methylxanthine	H	H	CH_3
Xanthine	H	H	H

Figure 4. Chemical structure of caffeine and related methylxanthines.

The presence of proanthocyanidins in the coffee pulp has been highlighted by various authors (González De Colmenares et al 1998). However the determination of the nature and the proportion of constitutive units of proanthocyanidins present in the coffee pulp were never described before. A thorough knowledge of the structure of the tannins present in the coffee pulp could make it possible to develop suitable methodologies in order to degrade them, in order to use the pulp as animal feed. However, coffee pulp also contains caffeine (approximately 1% dry weight), one of the pharmacologically active molecules most consumed in the world.

3. STUDIES ON THE DEGRADATION OF CAFFEINE BY FILAMENTOUS FUNGI

Nutritious substances present in coffee pulp are closely associated with caffeine and phenolic compounds, molecules known to have antiphysiological and antinutritional effects (Ulloa Rojas et al 2002). In fact, the development of techniques to decaffeinate has interested many research teams in

the world. Our research has been focused towards the use of microorganisms to eliminate caffeine present within coffee pulp. It was known that filamentous fungi were able to grow in the presence of caffeine as sole source of nitrogen. Coffee pulp being a very complex substrate, it was considered useful to begin research with a clean and controllable model system to study the biological breakdown of caffeine by fungi (Aquiahuatl 1992; Roussos et al 1989). Initially, nearly 300 fungal strains were isolated from samples taken from the sites of production of coffee in Mexico. From these, eight strains were selected to study the degradation of caffeine in solid-state fermentation (SSF) using coffee pulp as support and substrate (Aquiahuatl et al 1988; Roussos et al 1989; Perraud-Gaime 1995). Two of these fungal strains, *Aspergillus* V12A25 and *Penicillium* V33A25 appeared to be able to completely degrade the caffeine present in coffee pulp (Perraud-Gaime 1995). However, the degradation products were unknown and moreover, certain degradation products of caffeine (Figure 4) such as theophilline or theobromine have the same antiphysiological characteristics as caffeine. It was, therefore, necessary to know the different steps involved in the degradation of caffeine by filamentous fungi. Moreover, was the degradation pathway the same for filamentous fungi in general? The ability of several filamentous fungi to degrade caffeine as well as the corresponding dimethylxanthines was, therefore, assessed. Twenty different strains were tested for their ability to use caffeine as the sole source of nitrogen in liquid media. The kinetics of caffeine and related dimethylxanthines degradation were established for all caffeine-degrading strains. In each experiment, degradation products were characterized. Out of twenty strains tested for their ability to grow in a medium containing caffeine as the sole nitrogen source (CS medium), only seven strains were able to grow (Table 2). Their ability to degrade caffeine was checked by HPLC analysis of the culture supernatant. In each case, there was caffeine degradation only if fungal growth was observed. The seven strains were not able to grow when caffeine was used as a sole source of carbon and nitrogen. In later experiments, only the seven strains able to grow on CS-medium were used. The strains were quantitatively tested for their ability to degrade caffeine, theophylline, theobromine and paraxanthine as a sole nitrogen source.

Table 2. Caffeine degradation by the selected strains

Code number	G. species	% degradation at 48h	V mean (mg/L.h.)
V12A25	*A. tamarii*	67.2	53.6
V33A25	*P. commune*	56.8	35.0
C25A35	*A. fumigatus*	17.6	14.7
V29A25	*P. commune*	67.7	48.0
V14A35	*P. commune*	46.0	39.2
C28B25	*A. niger*	16.1	30.7
C7A25	*P. commune*	61.6	52.1

A high concentration of caffeine in caffeine degradation experiments (4 g/L after 48 hours of culture at 1 g/L) to be able to see as many intermediates as possible. *A. tamarii* (V12A25) and all *P. commune* strains showed a good ability to degrade caffeine whereas *A. niger* (C28B25) and *A. fumigatus* (C25A35) were less effective (Table 1, Figure 5). *A. niger* degradation profile (C28B25) was the

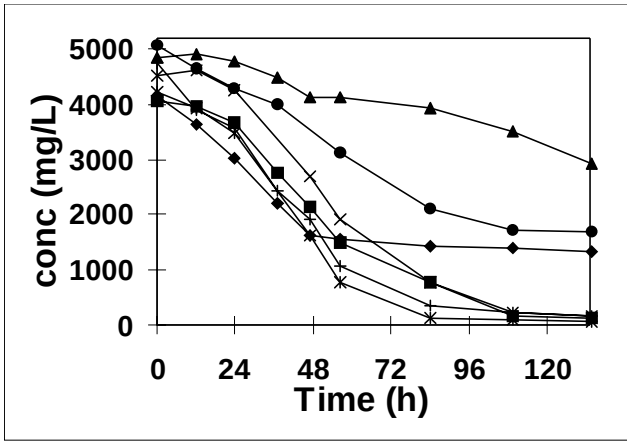


Figure 5. Caffeine degradation by the seven selected strains.
● C28B25, ◆ V12A25, △ C25A35, ✕ V14A35, ✳ V29A25, + C7A35, ■ V33A25

same as that of *A. fumigatus* (C25A35) (Figure 6, B). *P. commune* degradation profile (V14A35) (Figure 6, A) is representative of all other strains except *A. tamarii*. In this case, biomass production was higher than with other strains, sucrose disappeared from culture medium after 48 hours of culture and no further degradation was noted at this incubation time (Figure 5).

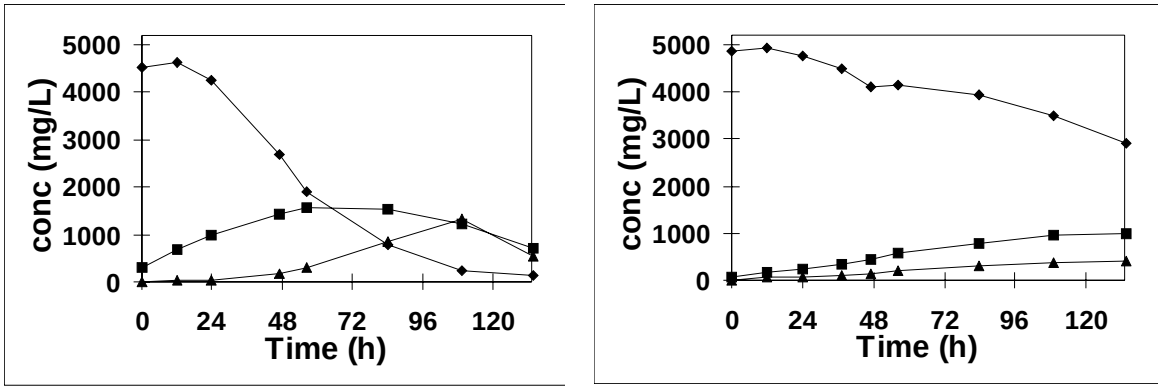


Figure 6. Caffeine degradation and appearance of products. A (TOP), V14A35. B (Bottom), C25A35
◆ caffeine, ■ theophylline, △ 3-methylxanthine.

Figure 6A (top) showed that theophylline appeared first followed by 3-methylxanthine. These two compounds represented up to 60% of initial caffeine present during caffeine degradation by *P. commune* (V14A35). Theobromine and paraxanthine were detected in the culture medium at concentrations of about 40 mg/L. Trace amounts of 1-methylxanthine were also detected. In all HPLC assays, no trimethyluric acid or dimethyluric acids were detected.

From all these experiments, it was concluded that the first steps of caffeine degradation in filamentous fungi consisted of demethylation reactions. Demethylations in position 1 and 7 can occur each time a methyl group is present in this position but the 7-demethylation seems to be preferentially used. Ina (1971) identified xanthine as a degradation product of caffeine by a strain of *A. niger*. It would be interesting to further identify the products resulting from caffeine degradation of our seven strains.

It is difficult to say whether caffeine degradation in filamentous fungi is due to one or more enzymes and whether these enzymes are the same as those present in bacteria. In bacteria, caffeine degradation is led by at least two enzymes and one of these enzymes has been purified. Answers to these questions could come from attempts presently made to purify the enzyme(s) implicated in the first steps of caffeine degradation. Such attempts are presently underway.

The analysis of antiphysiological compounds present in coffee pulp has been greatly advanced in the past years, however, much yet remains to be done in order to detoxify coffee pulp and obtain a "new" and cheap feed source for animal feed. It is to be noted that along the way, during the identification process, for example of the phenolic compounds present in coffee pulp, new molecules with biotechnological potential have been identified (catechin or taxifolin). It may well be a reality that in the next few years, coffee pulp, actually a waste product with just about no added value could turn out to be a highly-sought product with a high added value in the biotechnological world.

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