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Synthesis of copper nanoparticles using grape stalk and spent coffee extract

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Abstract

Nanotechnology has great potential in improving waste water treatment. Physical and chemical methods of obtaining nanoparticles have already been explored a great deal. They have however some drawbacks such as hazardous reaction conditions and expensive reagents. Green nanotechnology is a new way of producing nanoparticles which has a less drastic reaction and is eco-friendly. Green synthesis of nanoparticles can be achieved by using for example microorganisms or plant extracts. In this study, biosynthesis of metal nanoparticles was done using grape stalk (waste from winery) and spent coffee (after production of soluble coffee) waste. These agrofood wastes contain a high concentration of reducing agents such as polyphenols and sugars that could be adequate to reduce the metals in solutions to zero-valent and obtain the nanoparticles. The grape stalk and spent coffee extracts were obtained using Milli-Q water as a solvent. Temperature, contact time, ratio solvent/waste and particle size were conditions that were examined to obtain the highest concentration of reducing agents possible. The concentration of reducing agents in the extracts was analysed. The extract was then added to a synthetic copper solution to obtain the nanoparticles. The effects of temperature, pH, contact time and ratio metal/extract were examined. The nanoparticles were characterized by using scanning electron microscopy (SEM) coupled with EDX and UV-Vis absorption.

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Part 1: Theory

Nanotechnology is the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. Ultrafine particles are the same as nanoparticles and are ranged between 1 and 100 nanometers in size, fine particles are sized between 100 and 2,500 nanometers, and coarse particles cover a range between 2,500 and 10,000 nanometers. Nanoparticle research (mostly metals) is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical (Mittal et al. 2013), optical and electronic fields (Iravani 2011)

1 'Green' nanotechnology

The methods for making nanoparticles generally involve a 'top-down' or a 'bottom-up' approach (see figure 1). With the top-down method, nanoparticles are synthesized by size reduction from a starting material. This size reduction is achieved by various physical and chemical techniques. In the bottom-up approach, the nanoparticles are built from smaller particles or molecules. The building blocks are formed first and then assembled to give the final particle. The bottom-up approach relies on chemical and biological methods of production. 'Green' or biological synthesis of nanoparticles is a form of the bottom-up method of nanoparticle production (Mittal et al. 2013). The biological synthesis starts with the reduction of a metal (usually an aqueous solution) to the zero-valent metal or an oxide of the metal. Plants, microorganisms, fungi and algae can be used for the reduction (whole organisms or extracts of the organism/plant). The nanoparticles that are formed after reduction can be stabilised (capped) by the same compounds that are responsible for the reduction of the metal salt. The stabilisation will prevent the formation of aggregates.

Nanoparticles have been produced physically and chemically for a long time, but their synthesis remains expensive and involves the use of hazardous chemicals. That is why the 'green' environmentally friendly synthesis has become more popular over the years. It is eco-friendly, simple, cheap and can easily be upscaled. Different biological systems of bacteria such as Enterobacteria (Shahverdi et al. 2007) and Rhodopseudomonas capsulata (He et al. 2007), funghi such as Fusarium oxysporum (Ahmad et al. 2003) and plants such as Argemone mexicana (Kharissova et al. 2013) can be used as reducing and capping agents. Of these three, the plants are the simplest and the cheapest way for bioreduction because they don't have to be bought or cultured in advance like microorganisms.

2 Plant extract

Plant extracts contain various reducing agents (alkaloids, phenolic compounds, terpenoids) and co-enzymes. Using plant extracts for the production of nanoparticles is easier than using whole plants and plant tissues. It is environmentally friendly because there are no hazardous chemicals involved. The compounds in the extracts may act as both a reducing agent and a stabilising agent (capping) in the synthesis of the nanoparticles (see Figure 2). First the metal (usually a salt) is reduced to zero-valent metal or metal oxides. Then the particles start growing. The growth of the particle depends on the nucleation and crystal growth rate. Finally the particle is stabilized by capping agents (sugars, phenols,…) present in the extracts (Mittal et al. 2013). The type of plant is known to influence the characteristics of the nanoparticles because they contain different concentrations and mixtures of organic reducing agents. Examples of plants that have already been used for the production of metal nanoparticles are cloves (Subhankari & Nayak, 2013), *Oecimum sanctum* leaves (Kulkarni & Kulkarni, 2013), orange (Castro et al. 2013) and many more. In this paper extracts of spent coffee waste and grape stalk waste will be investigated as possible promotors of metal nanoparticles.

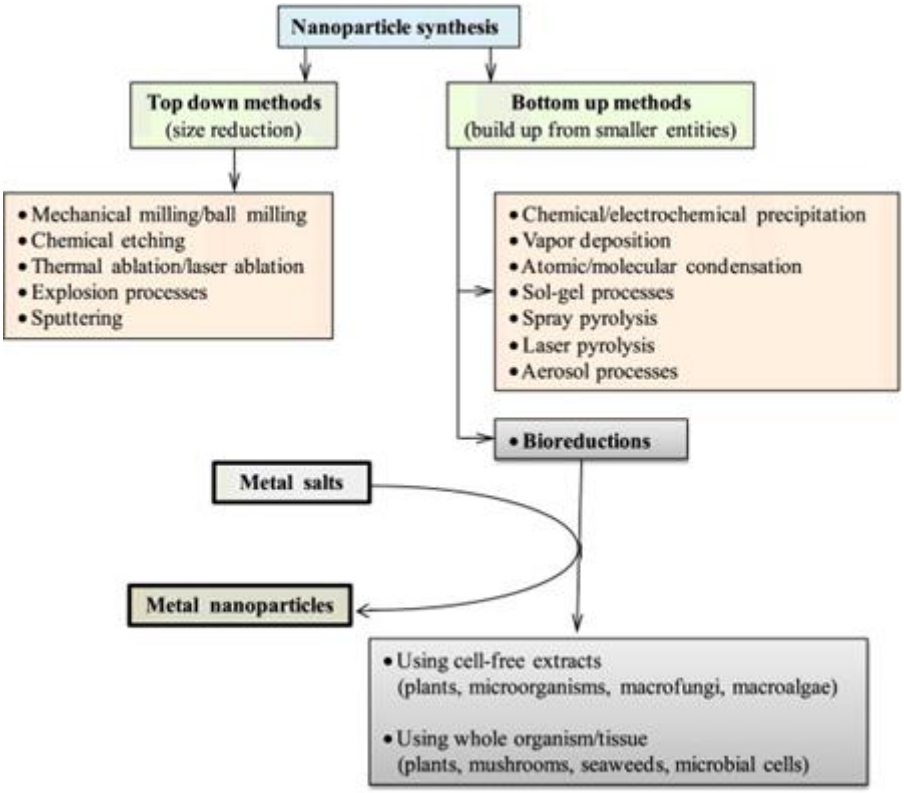


Figure 1: top-down and bottom-up nanoparticle synthesis (Mittal, Chisti, & Banerjee, 2013)

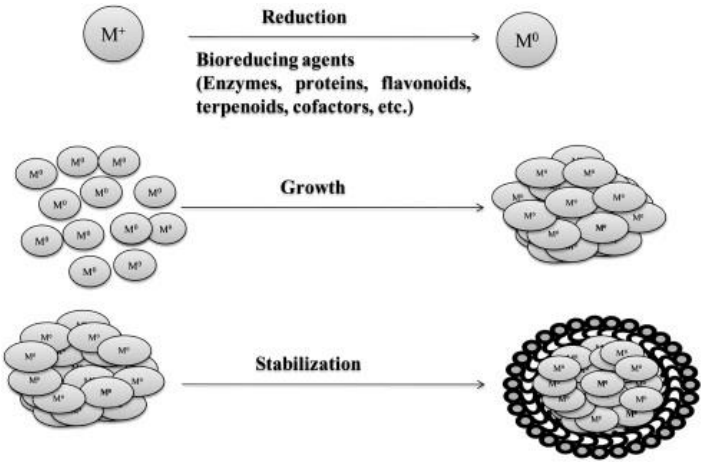


Figure 2: biosynthesis of nanoparticles (Mittal et al. 2013)

2.1 Spent coffee

Spent coffee waste is the pressed and dried solid residue left after coffee extraction for the production of soluble coffee (see figure 3). It represents 50% of the mass of coffee feedstock, so a large amount of waste is generated every year. This waste is used for different purposes such as composting, bioenergy production and mushroom growth. This waste contains organic compunds such as cellulose, fatty acids, lignin, sugars, polyphenols and tannins that may be used as both reducing agents and capping agents (D. Pujol et al. 2013).

Figure 3: coffee waste (Gerits, 2015)

2.2 Grape stalk

Grape stalk is the skeleton of grapes (see figure 4). They are obtained from stripping operations during wine production. Grape stalks represent the main by-products of vineyards. Since grape stalks have to be disposed in a landfill or biologically treated, they become an environmental problem. The use of grape stalks as a resource for the production of new products has attracted a lot of attention over the years. It contains extractives (mostly polar compounds) such as polyphenolic compounds, sugars and tannins (David Pujol, Liu, et al. 2013) that may act as reducing agent and stabilizing agent at the same time. The chemical composition depends on different factors such as origin, time of harvest and grape varieties.

Figure 4: Grape stalk (Gerits, 2015)

2.3 Preparation of the extract

The preparation of a plant extract is very straightforward. Several authors were consulted to obtain information about the possible conditions needed to obtain the extract (see Table 1). In general, the plant is put into contact with water and stirred.

Table 1: conditions for obtaining extract from literature

Name plant	Weight (g)	solvent	Solvent volume (ml)	Time (min)	Temp (°C)	reference
Cloves (leaf)		Distilled water	10	2-3	70-80	(Subhankari & Nayak 2013)
Ocimum sanctum (leaf)	100	Deionized water	100	15	80	(Kulkarni & Kulkarni 2013)
Cassia auriculata (flower)	50	Distilled water	100	60	100	(Ramesh et al. 2014)
Orange peel		Deionized water		30	100	(Castro et al. 2013)
Citrus paradisi (peel)	10	Double distilled water	40	30	80	(Kumar et al. 2014)
Artabotrys odoratissimus (leaf)	25	Distilled water	500	5	100	(Umesh & Gajera 2014)
Garcinia mangostana	2.5	Distilled water	100	60	100	(Rajakannu et al. 2015)
Salvia officinalis, Lippia citriodora, Pelargonium graveolens, Punica granatum	10	Double distilled water	50			(Elia et al. 2014)
Grape seeds, grape stalk, grape skin	3	Deionised water	50	2	60	(Krishnaswamy et al. 2014)
Ginkgo biloba L.	100	Double distilled water	500	30	70-80	(Nasrollahzadeh & Mohammad Sajadi 2015)

As can be seen in table 1, variables such as temperature (60-100°C), contact time (2-60 minutes) and ratio waste/solvent (1:1, 1:2,…) are variables studied by these different authors. These variables were therefore examined with the aim of obtaining the highest concentration of compounds in the extract.

3 Metal nanoparticles

Nanoparticles are of interest because of their size and large surface area in respect to volume ratio. This leads to both chemical and physical differences in their properties (mechanical, biological, sterical, …) compared to bulk of the same chemical composition. For example, the bending of bulk copper (wire, ribbon, etc.) occurs with movement of copper atoms/clusters at about the 50 nm scale. Copper nanoparticles smaller than 50 nm are considered super hard materials that do not exhibit the same malleability as bulk copper. The large surface area to volume ratio is responsible for the lower melting temperature of nanoparticles compared to the bulk material (Moores & Goettmann 2006).

Colloidal solutions of copper, gold and silver nanoparticles have interesting optical properties that rely on a strong absorption in the visible spectrum (Moores & Goettmann, 2006). Therefore the particles can be produced for a specific application.

Many different physical and chemical methods (etching, electrochemical precipitation,…) already exist for the production of metal nanoparticles but they are often expensive and hazardous for the environment (Iravani 2011). Therefore biological synthesis is environmentally benign, easy and cheap.

3.1 Application

Gold and silver nanoparticles are used in biomedicine as markers, sensors and antimicrobials (Mittal et al. 2013). They have a low toxicity to humans. Metal nanoparticles such as copper have great potential in the field of wastewater treatment as an adsorbent for contaminants because of their high specific surface area, tunable surface chemistry and because they are easy to reuse.

Conductive inks and pastes containing copper nanoparticles can be used as a substitute for very expensive noble metals used in printed electronics and displays (Lee et al. 2008). Gold nanoparticles immobilised in oxide matrixes are known to be active oxidation catalysts and devices using this technology are now commercialised in Japan for anti-odour systems (Moores & Goettmann, 2006).

3.2 Synthesis

For the synthesis of metal nanoparticles (zero-valent metal or metal oxides) different authors were consulted to obtain information about the conditions necessary for the synthesis (see table 2). In general, the plant extract is put into contact with an aqueous solution of the metal salt.

Table 2: conditions for obtaining nanoparticles

metal	Metal sol conc (M)	Solvent	Metal sol volume (ml)	Extract volume (ml)	pH	Temp (°C)	Time (min)	reference
CuSO4	0,001	Distilled water	5	5	1,89		60	(Subhankari & Nayak, 2013)

CuSO4.5H2O	0,001	Deionized water	100	1		20 (roomtemp)	720	(Kulkarni & Kulkarni, 2013)
HAuCl4	100 mg/L				2,7,10	20		(Castro et al. 2013)
AgNO3	100 mg/L				2,7,10	60		(Castro et al. 2013)
ZnSO4.7H2O	0,003	Milli-Q	10	3	8	75-80	180	(Kumar et al. 2014)
CuSO4.5H2O	0,001		170	30		95		(Umesh & Gajera 2014)
Zn(CH3COO)2.2H2O	0,02	Distilled water		0.25,0.5,1	12		120	(Sabir et al. 2014)
Zn(CH3COO)2		Deionized water		100	3,7-4	90	180	(Ain Samat & Md Nor 2013)
AgNO3	0,001	Distilled water	95	10		80	15	(Rajakannu & Shankar, 2015)
HAuCl4	0,1 g/l	Double distilled water	10	0,75		35-40	5	(Elia et al. 2014)
HAuCl4.3H2O	0,001	Deionized water	12	5				(Krishnaswamy et al. 2014)
CuCl2.2H2O	0,005	Deionized water	10	100	9		15	(Nasrollahzadeh & Sajadi, 2015)

As can be seen in table 2, temperature (20-95°C), pH (2-12), ratio metal/extract (1:1, 1:2,…), contact time (5-720 min) and agitation are parameters discussed by the authors. The influence of these parameters on the obained nanoparticles was examined.

4 Instrumental Analysis

For the instrumental analysis, several authors were consulted to obtain information about the instruments that were used for the analysis of both the extracts (see table 1) and the metal nanoparticles (see table 2). Techniques such as UV-Vis absorption spectroscopy and scanning electon microscopy (SEM) were used by

several authors for the analysis of the metal nanoparticles. The plant extracts contain organic compunds that could act as a reducing agent. To acquire information about the concentration of organic compounds present in the extracts, a total organic carbon analyzer was used.

4.1 UV-Vis spectroscopy

The intensity of light passing through a sample is compared to the intensity before it passes through the sample. Ultraviolet and visible light are energetic enough to promote outer electrons to higher energy levels. It is used as a quantitative method of analysis. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Lambert-Beer law. The basic parts are a light source, a diffraction grating in a monochromator to separate the different wavelengths of light and a detector (see figure 5).

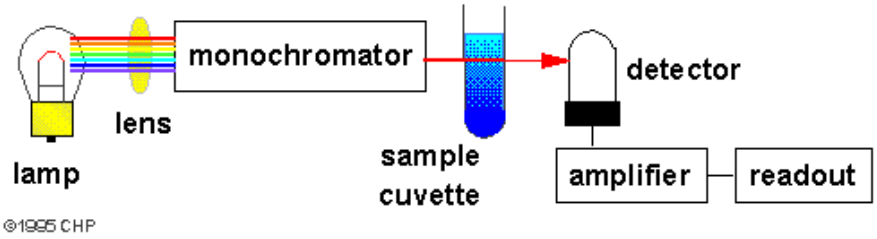


Figure 5: UV-Vis spectrofotometer (Brian 1996)

4.2 Scanning electron microscopy (SEM)

Energy-dispersive X-ray spectroscopy is first used for the elemental analysis of the sample. Its characterization capabilities are mainly due to the fundamental principle that each element has a unique atomic structure allowing a unique set of peaks in its X-ray emission spectrum.

SEM is a an electron microscope that produces images by scanning it with a focused beam of electrons. The electrons interact with the atoms in a sample. The electron beam's position is combined with the detected signal to produce an image. In a typical SEM, an electron beam is thermionically emitted from an electron gun fitted with a tungsten filament cathode. The electron beam is focused by condenser lenses to a spot of 0,4 to 5 nm in diameter. The beam passes through pairs of scanning coils which deflect the beam so that it scans in a raster fashion over an area of the sample surface.

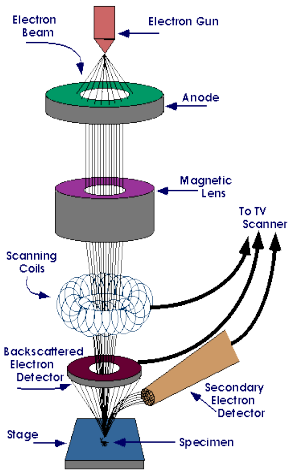


Figure 6: scanning electron microscope (Purdue university 2014)

When the primary electron beam interacts with the sample the electrons lose energy which results in the reflection of high energy electrons (backscatter), emission of secondary electrons and the emission of electromagnetic radiation. Each of these can be detected separately. The backscattered electrons may escape from great depths in the sample because of their high energy while the secondary electrons come from the surface of the sample (see figure 6).

4.3 Total organic carbon (TOC)

The total organic carbon is the difference between the total carbon (TC) and inorganic carbon (IC) values. For the determination of the TC, the sample is introduced into a combustion tube which is filled with an oxidation catalyst and heated to 680 ° C . The sample is burned in the combustion tube and TC components in the sample are converted into CO_2. The carrier gas (oxygen) , flowing with a flow rate of 150 ml / min through the combustion tube, carries the products of combustion of the sample to the electronic dehumidifier cooled to 1 ° C and dehydrates the gas by condensation. The depressed gas carries the products of combustion of the sample through a scrubber to remove or trap chlorine and other halogens . Finally the carrier gas delivers the products in a cell of a non -dispersive infrared sensor (NDIR), which detects carbon dioxide .

The IC measured by TOC analysis consists of the carbon containing carbonates and carbon dioxide dissolved in the water. The sample is acidified with a small amount of hydrochloric acid (to obtain a pH between 2 and 3) , all carbonates are converted into CO_2 based on the following reactions:

$Me_2CO_3 + 2\ HCl \rightarrow CO_2 + 2\ MeCl + H_2O$
$MeHCO_3 + HCl \rightarrow CO_2 + MeCl + H_2O$

The CO_2 formed and dissolved CO_2 in the sample are applied to bubbling (purge) air or nitrogen gas (since it does not contain carbon dioxide) in the sample. The kit reactor of IC in TOC-V is used to analyze the IC or purge the sample through a liquid reaction acidified with H_3PO_4 . The sample is injected into the reaction vessel and the IC in the sample is converted into CO_2 , which are volatilized by the process of purging with carrier gas and transported to the NDIR detector.

Figure 7: TOC analyzer Shimadzu (Shimadzu corporation 2015)

The main components of an NDIR sensor are an infrared source (lamp), a sample chamber or light tube, a light filter and an infrared detector (see figure 7). The IR light is directed through the sample chamber towards the detector. In parallel there is another chamber with an enclosed reference gas, typically nitrogen. The gas in the sample chamber causes absorption of specific wavelengths and this is measured by the detector to determine the gas concentration. The detector has an optical filter in front of it that eliminates all light except the wavelength that the selected gas molecules can absorb.The NDIR detection gives a signal that is analogous to a peak.

5 Background

The research group “Metalls i Mediambient” from the University of Girona has investigated several agrofood by-products as possible biosorbents for metal ions removal and the chemical characterization of the compounds (polyphenols, reducing sugars, tannins) present in by-products such as spent coffee (Pujol, et al., 2013) and grape stalk (Pujol, et al., 2013). Grape stalk (Liu et al. 2015) and exhausted coffee waste (David Pujol, Bartrolí, et al. 2013) proved to be effective in the reduction of chromium(VI) to chromium(III).

This final project is centered on the possible use of extracts obtained from spent coffee and grape stalk waste for the synthesis of copper nanoparticles.

6 Objectives

The objective of the present final project is the green synthesis of copper nanoparticles based on extracts of grape stalk and spent coffee waste.

To attain this objective it is necessary to achieve different specific objectives.

(1) Study of the role of different parameters on concentration of organic compounds in the extract
(2) Study of the significance of different parameters on the synthesis of copper metal nanoparticles

Part 2: Practical aspects

The first step is the preparation of spent coffee and grape stalk extracts. Milli-Q water was used as the solvent. It diffuses into the coffee and grape stalk and solublizes the compounds with similar polarity. Organic solvents such as ethanol and methanol have also previously been used but the choice to use water as a solvent is based on the fact that it is simple and no contamination from the solvent is present. The effect of temperature, contact time, particle size and ratio waste/solvent were examined and adjusted to get the highest aqueous concentration of reducing agents.

The second step is the synthesis of the copper metal nanoparticles. Copper sulfate and copper chloride were used for the aqueous metal solutions. The effect of temperature, pH, contact time, agitation and ratio metal/extract on the formation of the nanoparticles was examined.

7 Preparation and analysis of extracts

The extracts of spent coffee and grape stalk were obtained by putting them into contact with water under continuous stirring. The parameters (temperature, contact time, ratio waste/solvent) that were tested during this contact may affect the concentration of reducing compounds in the extract. The concentration of reducing agents and the total organic carbon (TOC) were analysed. A statistical analysis was calculated to determine which parameters had an effect on the concentration of reducing agents (see figure 8).

7.1 Materials

7.1.1 Reagents

Milli-Q water was used as a solvent. Gallic acid (98%) was bought from ACROS. Sodium carbonate anhydrous (reagent grade) and the Folin-Ciocalteu reagent were bought from Scharlau. Copper(II) sulfate pentahydrate (reagent grade), sodium thiosulfate pentahydrate (reagent grade), potassium iodide (reagent grade), potassium sodium tartrate tetrahydrate (extra pure) and sodium hydroxide 1M were bought from Scharlau. Sulfuric acid (95-98%) was bought from ROMIL LTD.

7.1.2 Instruments

Magnetic stirrers from iseline MS-H-Pro were used for heating and agitation. For filtering, a büchner filter and cellulose paper were used. A Shimadzu UV-160A and UVmini-1240 UV-Vis spectrophotometer were used for the analysis of the total phenolic content. A Gilson sample changer with syringe pump was used to prepare the gallic acid standards and the dilution of the samples for the folin method. Milli-pore Millex HN Nylon filters (0,45 µm) and a Shimadzu TOC-VCSH analyzer were used for the TOC determination.

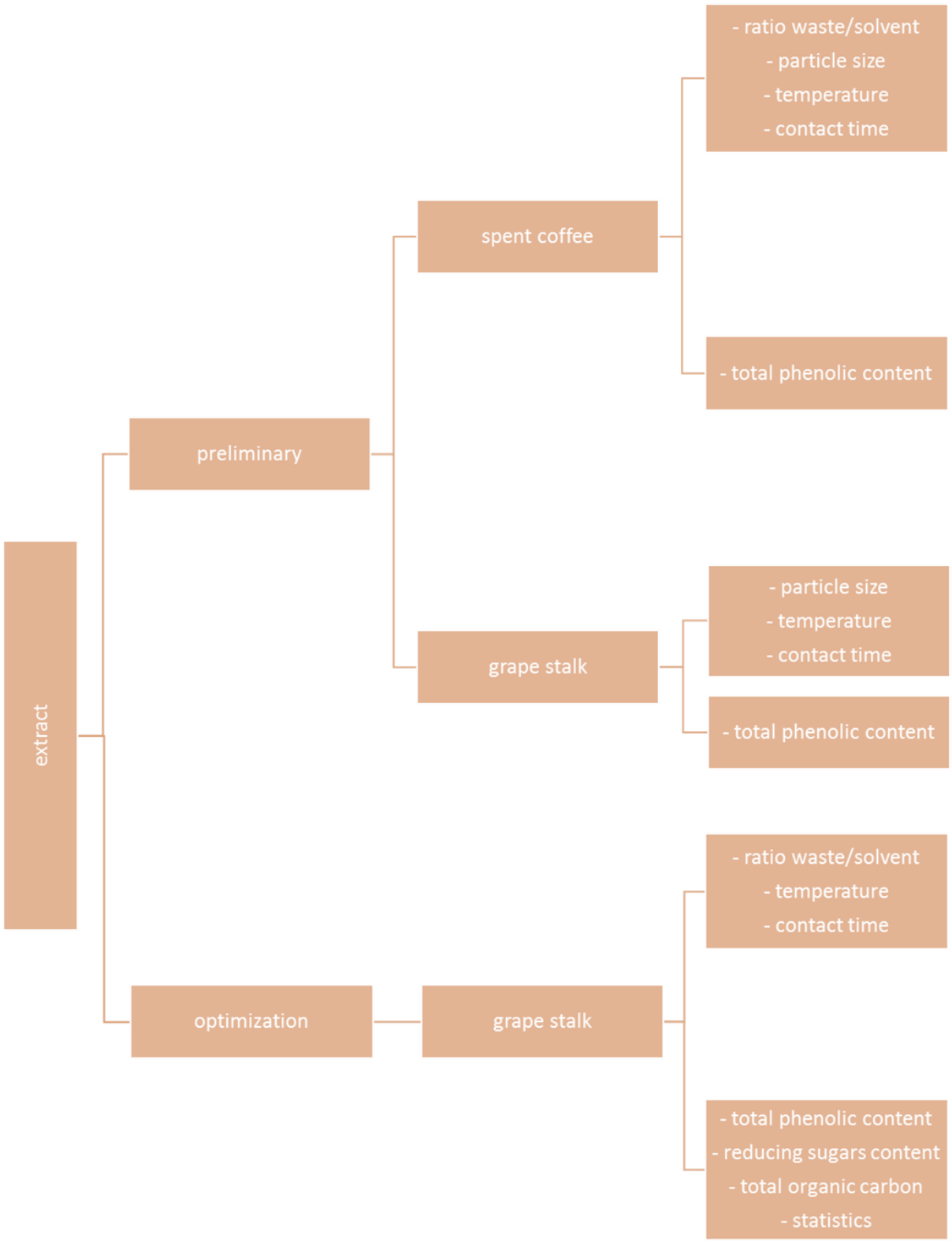


Figure 8: review of all variables examined and analysis performed of the grape stalk and spent coffee extracts

7.2 Method

7.2.1 Preliminary research on preparation of the extracts

For the preliminary research, several authors were consulted to obtain information about the conditions needed to prepare the extracts. The parameters ratio waste/sovent, temperature and contact time examined by these authors (see table 1) and an additional parameter of particle size were tested in the preparation of extracts based on spent coffee and grape stalk waste.

7.2.1.1 Spent coffee

For each preparation of spent coffee extract, the amount of spent coffee is approximately 3 grams. The variables examined are ratio waste/solvent volume (50 and 100 ml), particle size (100-250 µm, >1 mm), temperature (70°C, 100°C) and contact time (15, 30 minutes) (see table 3). Every preparation was done in duplicate.

Table 3: preliminary conditions for the preparation of coffee extracts

sample	Milli-Q (ml)	Coffee (g)	Particle size (µm)	Temp solvent (°C)	Time (min)
1a	50	3,001	100-250	70	30
1b	50	2,999	100-250	70	30
2a	100	2,999	100-250	70	30
2b	100	2,999	100-250	70	30
3a	50	3,000	>1 mm	70	30
3b	50	3,001	>1 mm	70	30
4a	100	2,999	>1 mm	70	30
4b	100	2,998	>1 mm	70	30
5a	50	2,999	100-250	100	30
5b	50	3,001	100-250	100	30
6a	100	3,000	100-250	100	30
6b	100	3,001	100-250	100	30

7a	50	3,002	100-250	70	15
7b	50	2,999	100-250	70	15
8a	50	3,000	100-250	100	15
8b	50	2,998	100-250	100	15

After the preparation, the extracts were filtered using a büchner filter with cellulose paper. The samples were kept in the fridge for further analysis.

7.2.1.2 Grape stalk

After the initial results of the concentration of reducing agents in the spent coffee extracts (see section 7.3.1.1), the decision was made to proceed with a ratio waste/solvent of 3 grams in 50 ml. All the grape stalk extracts were therefore prepared in 50 ml of Milli-Q and with approximately 3 grams of grape stalk. Variables tested are particle size (100-250 µm, >1 mm), temperature (70°C, 100°C) and contact time (5, 10 and 30 minutes) (see table 4).

Table 4: preliminary conditions for the preparation of grape stalk extracts

sample	Milli-Q(ml)	Grape stalk(g)	Particle size(µm)	Temp solvent(°C)	Time (min)
1a	50	3,002	100-250	70	30
1b	50	3,001	100-250	70	30
2a	50	2,999	>1mm	70	30
2b	50	3,002	>1 mm	70	30
3a	50	3,001	>1mm	100	30
3b	50	3,000	>1mm	100	30
4a	50	2,998	>1mm	70	10
4b	50	3,003	>1mm	70	10
5a	50	2,998	>1mm	70	5
5b	50	2,998	>1mm	70	5

After the preparation, the extracts were filtered using a büchner filter with Whatmann filter paper (0,25 µm). The samples were kept in the fridge for further analysis.

After analysis of the concentration of organic compounds it was decided to continue optimizing the parameters for the grape stalk extracts instead of the spent coffee extracts because the concentration was 10 times higher for the grape stalk extracts.

7.2.2 Optimization of grape stalk extracts

Because of the difficulty of filtration (small particles still in the filtrate and long filtration time) for the samples with particle size 100-250 µm, further research was performed with particle size 1-1,6 mm. This particle size has a smaller range than the preliminary research of >1 mm to better control the variable. The parameters tested were temperature (60, 70, 80, 100°C), contact time (15, 30, 60, 120 min) and ratio waste/solvent (50, 100 ml). The amount of grape stalk used (3 grams) and the particle size (1-1,6 mm) were kept constant (see Table 5).

Table 5: conditions for the preparation of grape stalk extracts

sample	Milli-Q(ml)	Grape stalk(g)	Particle size(µm)	Temp solvent(°C)	Time (min)
1a	50	3,001	1-1,6 mm	70	30
1b	50	2,999	1-1,6 mm	70	30
2a	100	3,000	1-1,6mm	70	30
2b	100	3,002	1-1,6 mm	70	30
3a	50	2,999	1-1,6mm	70	120
3b	50	3,001	1-1,6mm	70	120
4a	50	2,999	1-1,6mm	70	15
4b	50	2,998	1-1,6mm	70	15
5a	50	3,002	1-1,6mm	70	60
5b	50	2,999	1-1,6mm	70	60
6a	50	2,998	1-1,6mm	60	30
6b	50	2,997	1-1,6mm	60	30
7a	50	3,002	1-1,6mm	80	30
7b	50	2,997	1-1,6mm	80	30
8a	50	3,001	1-1,6mm	100	30
8b	50	3,002	1-1,6mm	100	30

9a	50	3,000	1-1,6mm	60	60
9b	50	2,998	1-1,6mm	60	60
10a	50	2,999	1-1,6mm	80	60
10b	50	2,999	1-1,6mm	80	60
11a	50	2,998	1-1,6mm	60	15
11b	50	2,998	1-1,6mm	60	15
12a	50	2,998	1-1,6mm	80	15
12b	50	2,997	1-1,6mm	80	15
13a	50	3,002	1-1,6mm	100	15
13b	50	2,997	1-1,6mm	100	15
14a	50	3,000	1-1,6mm	100	60
14b	50	2,999	1-1,6mm	100	60
15a	50	3,001	1-1,6mm	60	120
15b	50	2,997	1-1,6mm	60	120
16a	50	2,998	1-1,6mm	80	120
16b	50	2,997	1-1,6mm	80	120
17a	50	2,999	1-1,6mm	100	120
17b	50	3,001	1-1,6mm	100	120

7.2.3 Analysis of the extracts

For the analysis of the extracts, 4 techniques were used. The most important aspect of the extract is that it contains organic compounds that can reduce the metal in the solution. The techniques used focus on the determination of the amount of organic compounds present.

7.2.3.1 Total phenolic content

The Folin-Ciocalteu method was used to examine the total phenolic content (TPC) of the extract. Gallic acid was used as the standard. The Folin-Ciocalteu reagent contains a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and

phosphomolybdic acid ($H_3PMo_{12}O_{40}$) that is reduced to a mixture of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) by polyphenols present in the extract. The intensity of the blue color was measured using UV-Vis absorption at 760 nm after 2 hours.

Gallic acid (2000 mg/l) was used as a standard. 1 gram of gallic acid was weighed and dissolved in 500 ml of Milli-Q. 5 ml of the 2000 mg/l solution was diluted to 100 ml with Milli-Q (100 mg/l) . 10 grams of sodium carbonate was weighed and dissolved in 100 ml of Milli-Q to make the 10% wt solution. For the analysis of the coffee extracts, different low concentration standards (5, 10, 15, 20 mg gallic acid/l) were made. For the analysis of the grape stalk extracts, standards of 200, 400, 600, 800 and 1000 mg gallic acid/l were made.

For the coffee extracts, 1500 µl of sample or standard was added to 400 µl of Milli-Q, 200 µl of Folin reagent and 900 µl of sodium carbonate. For the grape stalk extracts, 100 µl of sample or standard was added to 3900 µl of Milli-Q, 600 µl of Folin reagent and 900 µl of sodium carbonate.

7.2.3.2 Reducing sugars

To determine the concentration of reducing sugars, a back titration using copper(II)sulfate, potassium iodide and thiosulfate was performed. The copper(II) was reduced by the sugars present in the extract.

$2\ (Cu^{2+} + e^- \rightarrow Cu^+)$

$3\ I^- \rightarrow I_3^- + 2\ e^-$

$2\ Cu^{2+} + 3\ I^- \rightarrow 2\ Cu^+ + I_3^-$

The excess of copper(II) will react with the iodide to obtain a coffee coloured solution. The thiosulfate is then added until the end point when the solution turns light purple. Starch is used as an indicator.

$I_3^- + 2\ e^- \rightarrow 3\ I^-$

$2\ S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2\ e^-$

$I_3^- + 2\ S_2O_3^{2-} \rightarrow 3\ I^- + S_4O_6^{2-}$

The difference between the amount of thiosulfate added to the blank and the sample, is equal to the amount of reducing sugars in g/l.

The solution of copper(II) sulfate was made by dissolving 41,92 grams of $CuSO_4.5H_2O$ and 10 ml of H_2SO_4 in 1 liter of deionized water. The alcaline-salts solution was made by dissolving 250 grams of sodium- and potassium tartrate in 400 ml of deionized water and adding 80 grams of NaOH dissolved in 400 ml of deionized water and bring it up to 1 liter. The potassium iodide solution was made by dissolving 300 grams of potassium iodide in 1 liter of deionized water and adding 100 ml of NaOH. The sulfuric acid solution (16%) was made by dissolving 87 ml of sulfuric acid (98%) in 1 liter of deionized water. The sodium thiosulfate solution was made by dissolving 13,777 grams of $Na_2S_2O_3.5H_2O$ and adding 50 ml of NaOH in 1 liter of deionised water.

For the titration, an erlenmeyer of 50 ml was filled with 10 ml of the copper sulfate solution (0,168 M). 5 ml of the alcaline-salt solution (0,886 M) and 2 ml of sample were added. The mixture was boiled for 2 minutes and cooled down as quickly as possible. After cooling down, 10 ml of potassium iodide (1,81 M) and 10 ml of sulfuric acid (16%) were added under stirring. This solution (brown) was then titrated with the thiosulfate solution (0,055 M) until the color changed to light purple. A few drops of starch solution (indicator) were added. The end point was reached when the color changed from light purple to light yellow.

7.2.3.3 Total organic carbon

The total organic carbon of samples 1-8 was analysed. 20 ml of the sample was filtered using Milli-pore Millex HN Nylon filters (0,45 µm) and a plastic syringe. The glass tubes were closed with a cap that contains a septum. The samples were then put into the TOC-VCSH for analysis.

7.2.3.4 Statistics

The statistical analysis program SPSS (version 23) for windows was used to determine which parameters (contact time, temperature) could have an influence on the concentration of polyphenols and reducing sugars in the extracts. Box plots and statistical tests (ANOVA and Bonferroni) were calculated.

7.3 Results and discussion

7.3.1 Preliminary results of the extracts

The extracts of spent coffee and grape stalk waste contain a certain concentration of reducing agents (organic compunds) such as polyphenols and reducing sugars. The concentration of polyphenols in the extracts was measured by UV-Vis spectroscopy.

7.3.1.1 Spent coffee extract

In order to determine the concentration of polyphenols in the extract, a calibration curve of a standard with known concentration was needed. Gallic acid was used as a standard for this analysis. The measurements of the standards and samples of the extracts were performed in duplicate.

The gallic acid standards (5, 10, 15, 20 mg/l) were measured by UV-Vis absorption at 760 nm (see table 6).

Table 6: absorbance for gallic acid standards used for polyphenols determination in spent coffee extracts

	concentration (mg gallic acid/L)	absorbance (A)
blank	0	0,012
blank	0	0,026
1	5	0,345
2	5	0,328
3	10	0,692
4	10	0,684
5	15	1,014
6	15	1,047
7	20	1,348
8	20	1,332

Correlation coëfficiënt	
R	0,999563495
R^2	0,999127181
Standard deviation	0,015590061
Observations	10

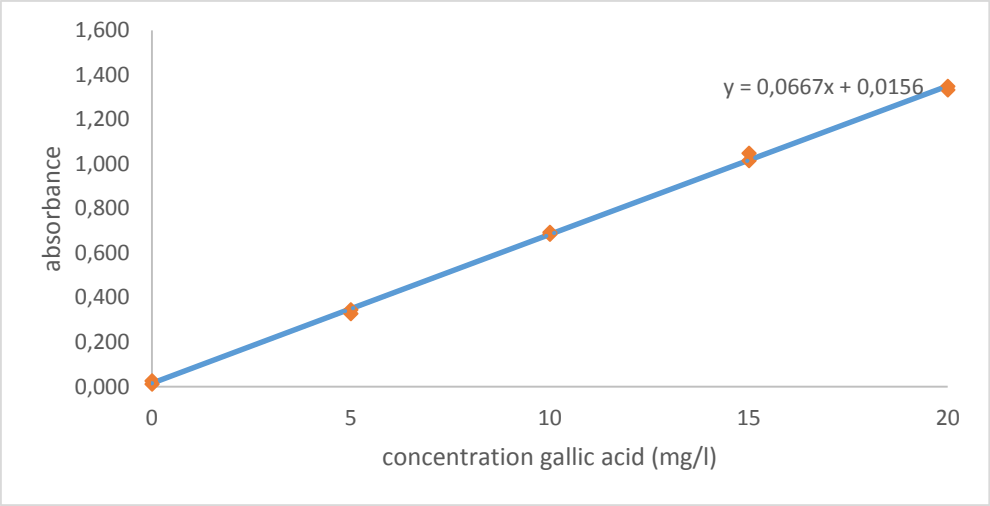


Figure 9: calibration curve for the concentration of gallic acid (mg/l) in the standards for spent coffee waste

The equation for the calibration curve is 0,0667 x + 0,0156 with a R^2 value of 0,99912 (see figure 9). The value of R^2 is sufficiently high to accept a linear fit.

The absorbance measured and the concentration calculated for the samples of coffee are seen in table 7. The samples were diluted (1 ml sample with 2 ml Milli-Q) to fit within the range of the calibration curve. The results of the diluted samples can be seen in table 7.

Table 7: concentration of polyphenols (mg gallic acid/l) for the samples of coffee

	Milli-Q (ml)	Coffee (g)	Particle size (µm)	Temp solvent (°C)	Time (min)	Polyphenols concentration (mg gallic acid/L)
1a	50	3,001	100-250	70	30	39,87
1b	50	2,999	100-250	70	30	38,16
2a	100	2,999	100-250	70	30	25,02
2b	100	2,999	100-250	70	30	26,94
3a	50	3,000	>1 mm	70	30	32,22
3b	50	3,001	>1 mm	70	30	35,24

4a	100	2,999	>1 mm	70	30	16,03
4b	100	2,998	>1 mm	70	30	16,57
5a	50	2,999	100-250	100	30	34,52
5b	50	3,001	100-250	100	30	45,27
6a	100	3,000	100-250	100	30	40,14
6b	100	3,001	100-250	100	30	38,96
7a	50	3,002	100-250	70	15	39,01
7b	50	2,999	100-250	70	15	37,71
8a	50	3,000	100-250	100	15	37,97
8b	50	2,998	100-250	100	15	41,61

The first variable examined was the particle size. The difference in concentration of polyphenols between samples 1-3 and samples 2-4 (see table 7) can be seen in figure 10. A first conclusion that can be made is that the smaller the particle size, the higher the concentration of polyphenols. This was expected because the contact between solvent and particle is better if the particle is smaller due to its larger surface area.

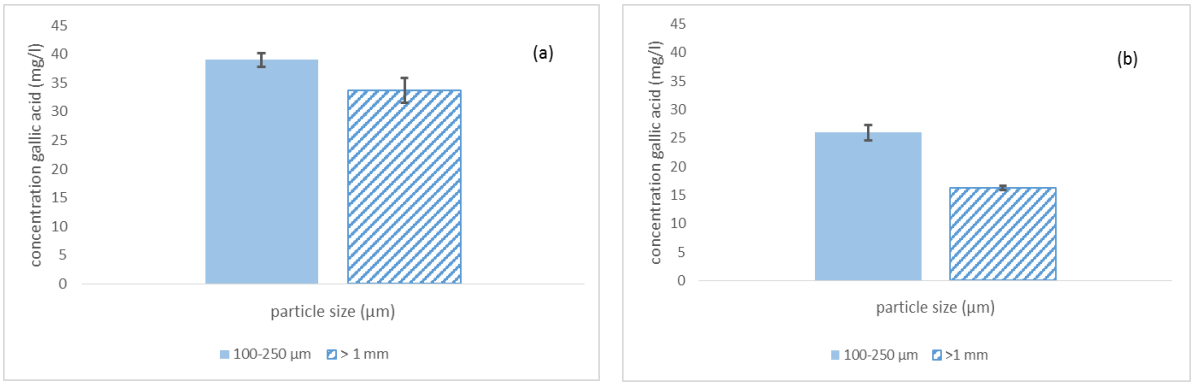


Figure 10: effect of particle size on the concentration of polyphenols of (a) 3 grams spent coffee in 50 ml Milli-Q at 70°C for 30 minutes and (b) 3 grams spent coffee in 100 ml at 70°C for 30 minutes

The effect of the temperature on the concentration of polyphenols between samples 1-5 and samples 2-6 can be seen in figure 11. This effect is smaller than the effect of the particle size. The increase in temperature doesn't seem to result in an increase of the concentration of polyphenols in 50 ml Milli-Q but the concentration increases at higher temperature in 100 ml. The overall concentration at 100°C is the same in 50 ml and 100 ml solvent. This can probably be explained by the fact that the spent coffee had already been introduced to boiling water during the process of making soluble coffee. A lot of compounds have already been extracted during this process.

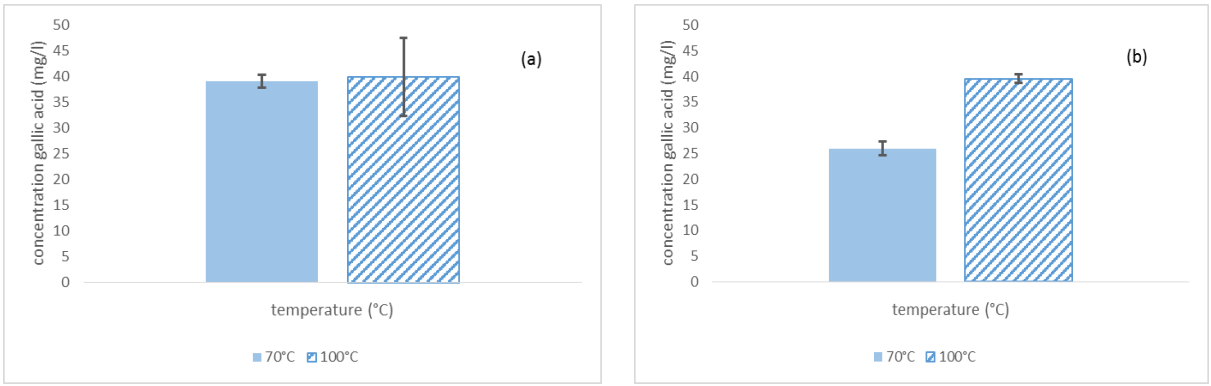


Figure 11: effect of temperature on the concentration of polyphenols of (a) 3 grams spent coffee in 50 ml Milli-Q with particle size 100-250 µm for 30 minutes and (b) 3 grams spent coffee in 100 ml Milli-Q with particle size 100-250 µm for 30 minutes

The next parameter examined was the contact time between samples 1-7 and samples 5-8 . The difference in concentration can be seen in figure 12.

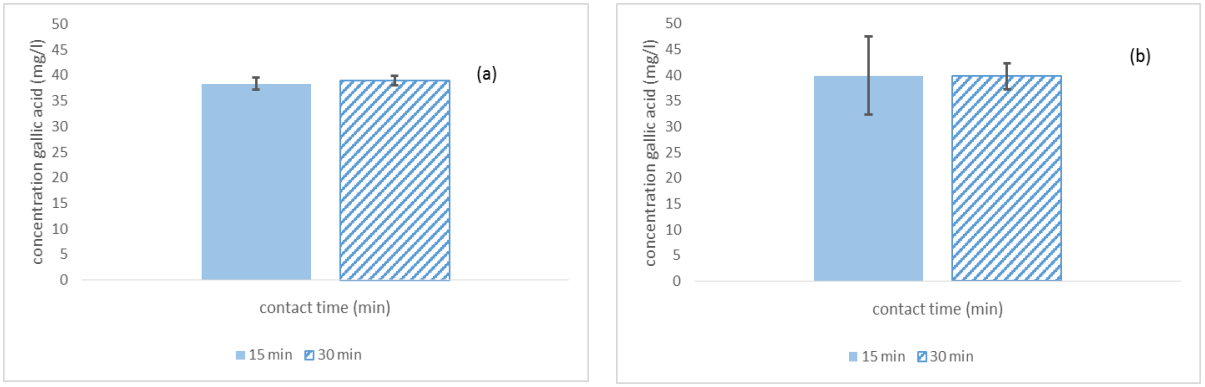


Figure 12: effect of contact time on the concentration of polyphenols of (a) 3 grams spent coffee in 50 ml Milli-Q with particle size 100-250 µm at 70°C and (b) 3 grams spent coffee in 50 ml Milli-Q with particle size 100-250 µm at 100°C.

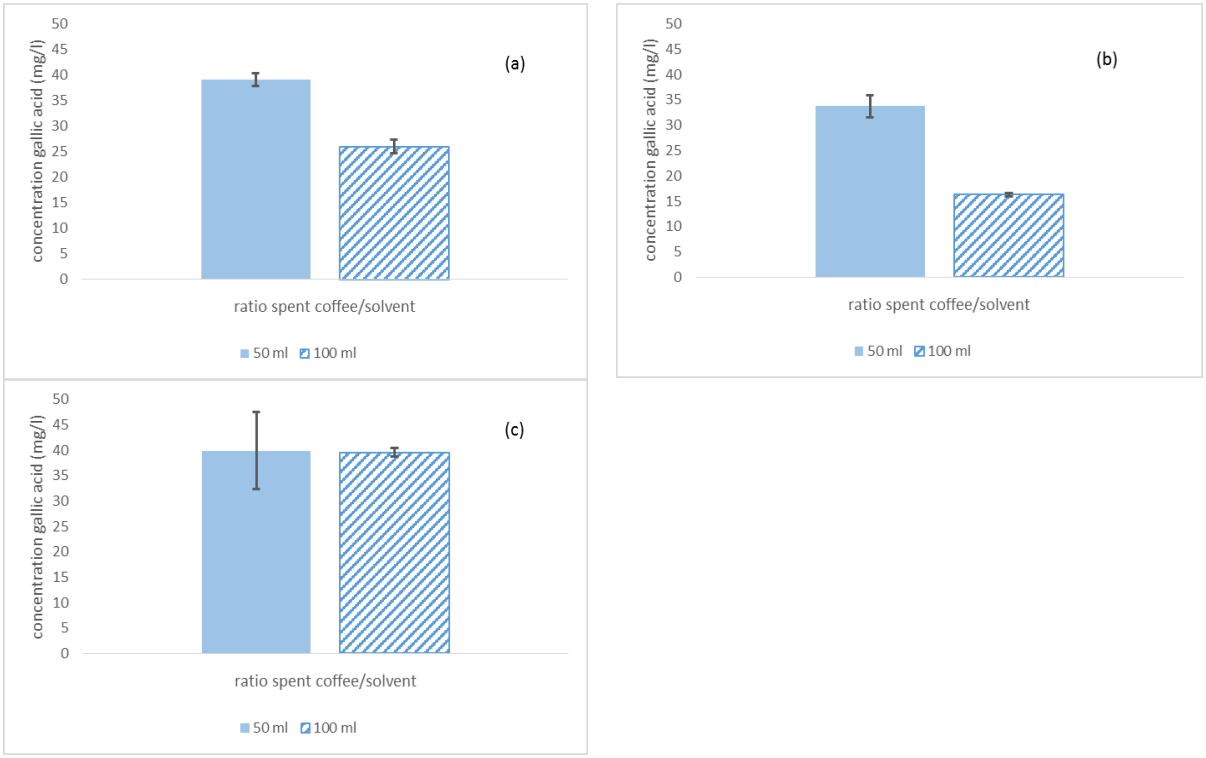


Figure 13: effect of ratio spent coffee/solvent volume on the concentration of polyphenols with (a) particle size 100-250 µm at 70°C for 30 minutes, (b) particle size > 1 mm at 70°C for 30 minutes, (c) particle size 100-250 µm at 100°C for 30 minutes

The resulting effect of the increase in contact time is very low (see figure 12). This can again be explained by the fact that a lot of compounds have already been extracted during the coffee making process. It seems that almost all the compounds are extracted after 15 minutes.

The last parameter tested was the ratio spent coffee/solvent volume between samples 1-2 and samples 3-4 and samples 5-6 (see figure 13). The concentration of polyphenols seems higher in 50 ml of solvent than in 100 ml of solvent. This was expected because a certain amount of polyphenols in a small volume should result in a higher concentration than in a higher volume (c = n/V).

7.3.1.2 Grape stalk extract

Gallic acid was again used as standard for the analysis of the calibration curve. The gallic acid standards of 200, 400, 600, 800 and 1000 mg/l and were measured using UV-Vis absorption at 760 nm. (see table 8). The measurements of the standards and samples of the extracts were performed in duplicate.

Table 8: absorbance for gallic acid standards used for polyphenols determination in grape stalk extracts

	Absorbance (A)	concentration (mg gallic acid/l)
blank	0,024	0
blank	0,009	0
1	0,444	200
2	0,445	200
3	0,874	400
4	0,887	400
5	1,295	600
6	1,298	600
7	1,761	800
8	1,742	800
9	2,165	1000
10	2,165	1000

Correlation coëfficiënt	
R	0,999919813
R^2	0,999839633
Standard deviation	0,010208097
Observations	12

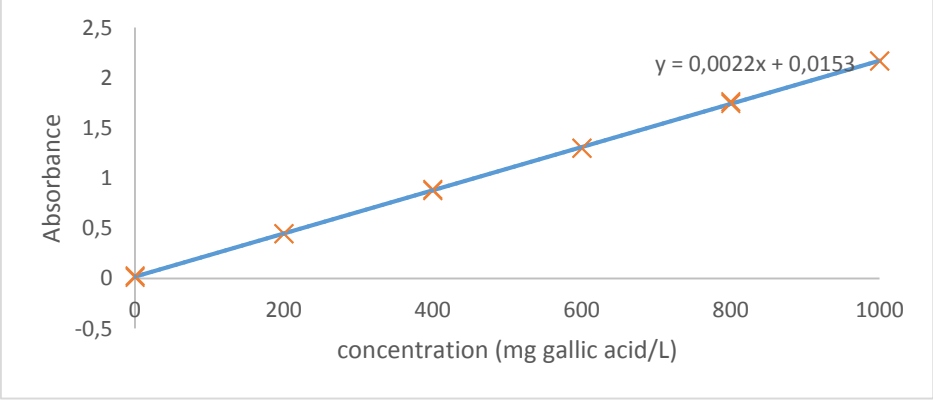


Figure 14: calibration curve for the concentration of gallic acid (mg/l) in the standards for grape stalk waste

The equation for the calibration curve is 0,0022x + 0,0153 with a R^2 value of 0,9998 (see figure 14). This value is good enough to accept a linear fit. The absorbance and the concentration of polyphenols (mg gallic acid/L) calculated for the obtained grape stalk extracts can be seen in table 9.

Table 9: concentration of polyphenols (mg gallic acid/l) for grape stalk extracts

	Milli-Q (ml)	Grape stalk (g)	Particle size (µm)	Temp solvent (°C)	Time (min)	Absorbance (A)	Polyphenols concentration (mg gallic acid/l)
1a	50	3,002	100-250	70	30	1,489	402,6
1b	50	3,001	100-250	70	30	1,338	361,8
2a	50	2,999	>1mm	70	30	1,332	360,2
2b	50	3,002	>1 mm	70	30	1,289	348,6
3a	50	3,001	>1mm	100	30	1,352	365,6
3b	50	3,000	>1mm	100	30	1,368	369,9
4a	50	2,998	>1mm	70	10	1,164	314,8
4b	50	3,003	>1mm	70	10	1,289	348,6
5a	50	2,998	>1mm	70	5	1,181	319,4
5b	50	2,998	>1mm	70	5	1,199	324,2

The variables of particle size, temperature and contact time were only examined between 2 or 3 samples to get a general idea of the possible effect on the concentration of polyphenols. The parameters with a possible significant effect were examined further during the optimization (see section 7.3.2).

Particle size (samples 1 and 2) was the first parameter examined. As can be seen in figure 15, the smaller particle size results in a higher concentration of polyphenols. This was expected because of better contact due to the larger surface to volume ratio resulting from a smaller particle size. It is the same result as with the coffee extract (see figure 10). The difficulty with the smaller particle size in grape stalk is filtering to separate the particles from the solution. There are still small particles present in the solution after filtering and it takes a long time because the pores of the filter become clogged.

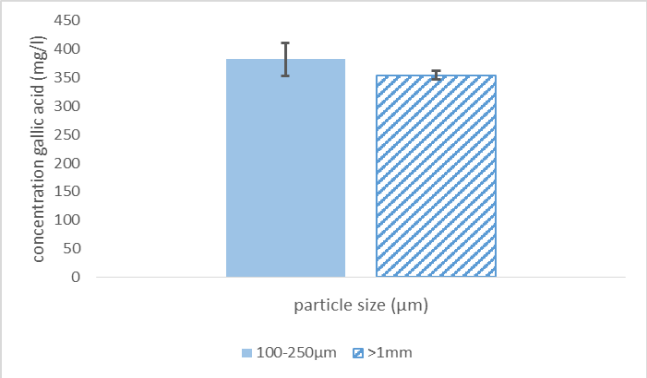


Figure 15: effect of particle size on the concentration of polyphenols for 3 grams of grape stalk in 50 ml Milli-Q at 70°C for 30 minutes.

As can be seen in figure 16 (samples 2 and 3), the concentration of polyphenols increases when the temperature increases. It is possible that if the temperature gets too high, the reducing compounds degrade or transform into other compounds. The general effect was expected because the higher the temperature, the better the solvent can diffuse into the waste to extract the compounds which results in a higher concentration. The temperature effect is more pronounced for grape stalk than for coffee (see figure 11).

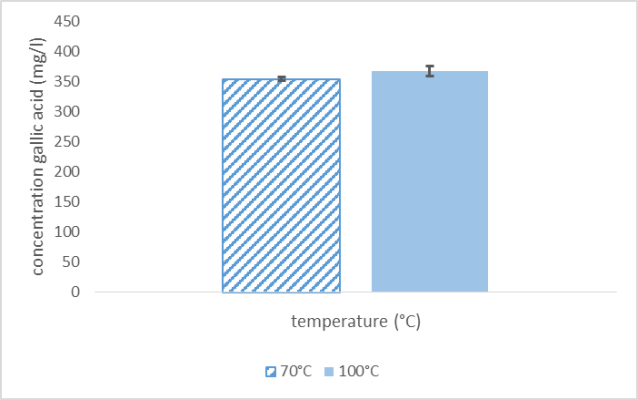


Figure 16: effect of temperature on the concentration of polyphenols for 3 grams of grape stalk in 50 ml Milli-Q with particle size >1 mm for 30 minutes

As can be seen in figure 17 (samples 1, 4 and 5), the contact time is a very important parameter in the case of grape stalk. The longer the contact time, the higher the concentration of polyphenols. It could be that not all compounds are extracted after 30 minutes. This is also to be expected. The longer the contact time, the more compounds can be extracted. The contact time needed to extract all the compounds (concentration reducing agents stays constant) has to be examined further.

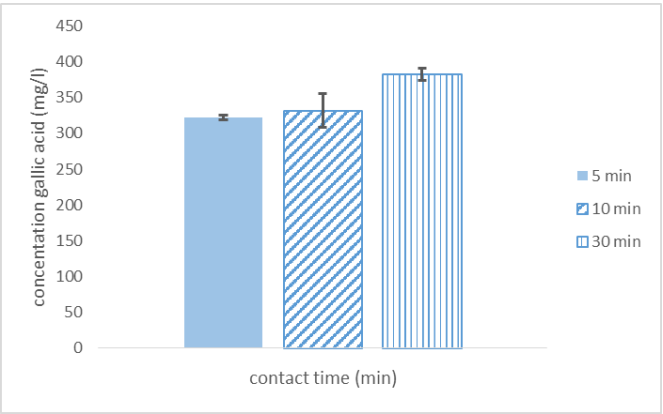


Figure 17: effect of contact time on the concentration of polyphenols for 3 grams of grape stalk in 50 ml Milli-Q with particle size >1mm at 70°C.

7.3.2 Results of optimization grape stalk extract

For the optimization of the grape stalk extracts, the parameters temperature, contact time and ratio waste/solvent were examined. Analysis of the total phenolic content, the reducing sugars content and the total organic carbon content was performed. A statistical analysis was used to prove or disprove the effect of the variables on the concentration of reducing agents in the extracts.

7.3.2.1 Total phenolic content

First a calibration curve was calculated using gallic acid standards of 200, 400, 600, 800, 1000 mg/l. The results of the concentration of polyphenols in the obtained grape stalk extracts can be seen in table 10. The amount of grape stalk (3 grams) and the particle size (1-1,6 mm) are kept constant.

Table 10: concentration of polyphenols (mg gallic acid/l) for grape stalk extracts

	Milli-Q(ml)	Grape stalk(g)	Particle size(µm)	Temp solvent(°C)	Time (min)	Polyphenols concentration (mg gallic acid/l)
1a	50	3,001	1-1,6 mm	70	30	673,3
1b	50	2,999	1-1,6 mm	70	30	673,3
2a	100	3,000	1-1,6mm	70	30	412,1
2b	100	3,002	1-1,6 mm	70	30	391,7
3a	50	2,999	1-1,6mm	70	120	765,5
3b	50	3,001	1-1,6mm	70	120	769,3
4a	50	2,999	1-1,6mm	70	15	587,8
4b	50	2,998	1-1,6mm	70	15	525,4
5a	50	3,002	1-1,6mm	70	60	714,1
5b	50	2,999	1-1,6mm	70	60	714,1
6a	50	2,998	1-1,6mm	60	30	612,3
6b	50	2,997	1-1,6mm	60	30	519,5
7a	50	3,002	1-1,6mm	80	30	758,4
7b	50	2,997	1-1,6mm	80	30	739,1

8a	50	3,001	1-1,6mm	100	30	743,9
8b	50	3,002	1-1,6mm	100	30	821,2
9a	50	3,000	1-1,6mm	60	60	697,2
9b	50	2,998	1-1,6mm	60	60	690,7
10a	50	2,999	1-1,6mm	80	60	982,5
10b	50	2,999	1-1,6mm	80	60	937,2
11a	50	2,998	1-1,6mm	60	15	542,3
11b	50	2,998	1-1,6mm	60	15	510,1
12a	50	2,998	1-1,6mm	80	15	799,8
12b	50	2,997	1-1,6mm	80	15	695,2
13a	50	3,002	1-1,6mm	100	15	867,8
13b	50	2,997	1-1,6mm	100	15	917,6
14a	50	3,000	1-1,6mm	100	60	1029
14b	50	2,999	1-1,6mm	100	60	1019
15a	50	3,001	1-1,6mm	60	120	749,1
15b	50	2,997	1-1,6mm	60	120	763,8
16a	50	2,998	1-1,6mm	80	120	901,9
16b	50	2,997	1-1,6mm	80	120	969,1
17a	50	2,999	1-1,6mm	100	120	958,2
17b	50	3,001	1-1,6mm	100	120	1072

The first variable examined was the ratio waste/solvent volume (see figure 18). The concentration of polyphenols is higher in 50 ml solvent than in 100 ml solvent.

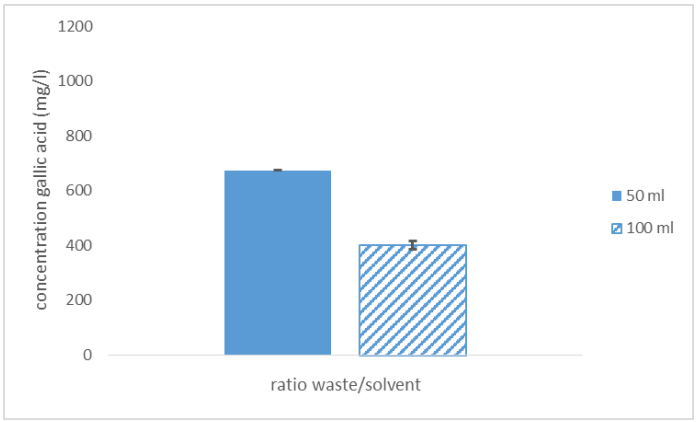


Figure 18: effect of ratio waste/solvent on concentration of polyphenols for 3 grams of grape stalk at 70°C for 30 minutes.

The next variable examined was the contact time at constant temperature. There seems to be a rise in the concentration of polyphenols when a longer contact time is applied (see Figure 19). At 120 minutes the concentration starts to decrease at 80°C and 100°C. This is because certain compounds transform or degrade or because all of the reducing agents have been extracted. In general, it confirms what was expected. The longer the contact time, the more compounds are extracted.

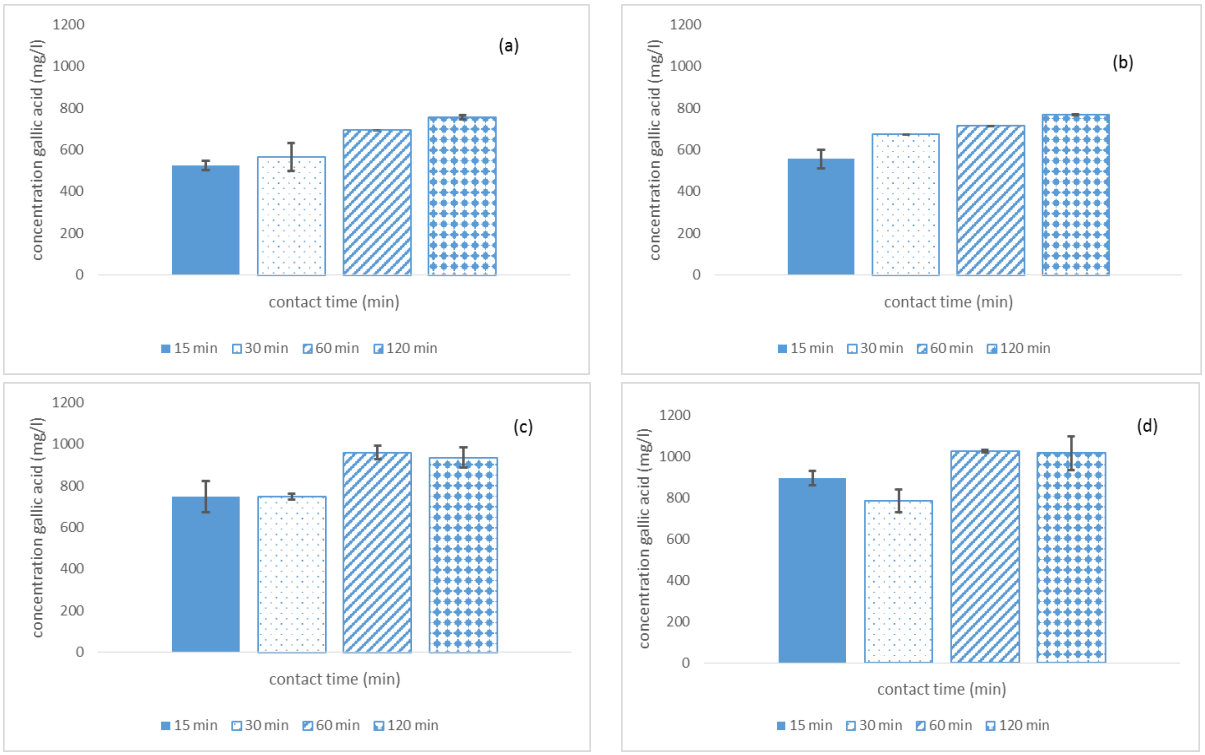


Figure 19: effect of contact time on concentration of polyphenols wtih particle size 1-1,6 mm at constant temperature of (a) 60°C , (b) 70°C, (c) 80°C, (d) 100°C.

The last variable was the temperature at constant contact time. It seems the higher the temperature, the higher the concentration of polyphenols in the extract is (see figure 20). The difference in concentration between 60 and 70°C is smaller than the concentration between 70 and 80°C. The difference in concentration between 80 and 100°C is also smaller than between 70-80°C. It was expected that the concentration of polyphenols would be higher with higher temperatures because the diffusion of the solvent into the grape stalk is better.

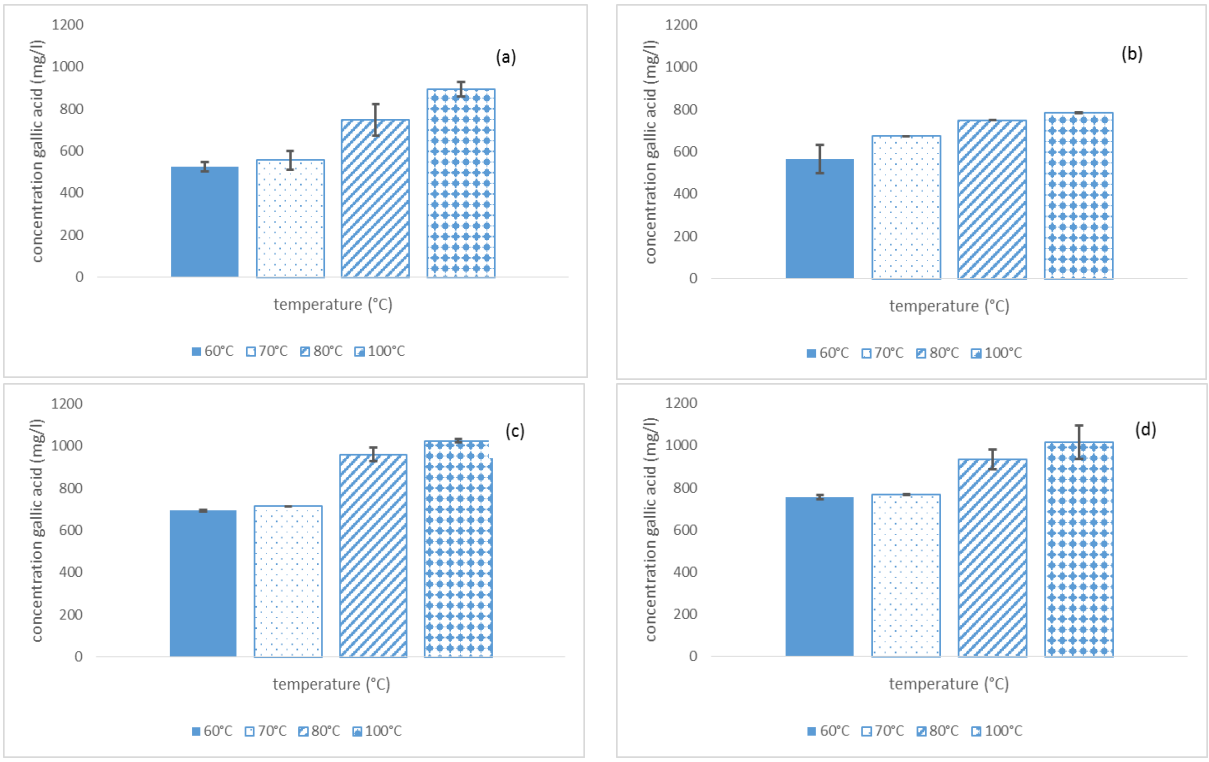


Figure 20: effect of temperature on concentration of polyphenols with particle size 1-1,6 mm at constant contact time of (a) 15 minutes, (b) 30 minutes, (c) 60 minutes and (d) 120 minutes.

7.3.2.2 Reducing sugars content

The concentration of reducing sugars in the coffee extracts was too low to be measured. The concentration of reducing sugars in the grape stalk extracts can be seen in Table 11.

Table 11: concentration of reducing sugars in the grape stalk extracts

	Milli-Q(ml)	Grape stalk(g)	Particle size(µm)	Temp solvent(°C)	Time (min)	Concentration (g/l)
1a	50	3,001	1-1,6 mm	70	30	3,2
1b	50	2,999	1-1,6 mm	70	30	3,4
2a	100	3,000	1-1,6mm	70	30	3,8
2b	100	3,002	1-1,6 mm	70	30	4,0
3a	50	2,999	1-1,6mm	70	120	3,0
3b	50	3,001	1-1,6mm	70	120	3,2
4a	50	2,999	1-1,6mm	70	15	3,5
4b	50	2,998	1-1,6mm	70	15	3,7

5a	50	3,002	1-1,6mm	70	60	3,5
5b	50	2,999	1-1,6mm	70	60	3,3
6a	50	2,998	1-1,6mm	60	30	3,4
6b	50	2,997	1-1,6mm	60	30	3,2
7a	50	3,002	1-1,6mm	80	30	3,3
7b	50	2,997	1-1,6mm	80	30	3,1
8a	50	3,001	1-1,6mm	100	30	3,7
8b	50	3,002	1-1,6mm	100	30	3,8
9a	50	3,000	1-1,6mm	60	60	3,4
9b	50	2,998	1-1,6mm	60	60	3,6
10a	50	2,999	1-1,6mm	80	60	3,1
10b	50	2,999	1-1,6mm	80	60	3,0
11a	50	2,998	1-1,6mm	60	15	3,2
11b	50	2,998	1-1,6mm	60	15	3,3
12a	50	2,998	1-1,6mm	80	15	3,1
12b	50	2,997	1-1,6mm	80	15	3,2
13a	50	3,002	1-1,6mm	100	15	3,6
13b	50	2,997	1-1,6mm	100	15	3,4
14a	50	3,000	1-1,6mm	100	60	3,7
14b	50	2,999	1-1,6mm	100	60	3,8
15a	50	3,001	1-1,6mm	60	120	3,9
15b	50	2,997	1-1,6mm	60	120	3,8
16a	50	2,998	1-1,6mm	80	120	3,9
16b	50	2,997	1-1,6mm	80	120	4,0

17a	50	2,999	1-1,6mm	100	120	3,2
17b	50	3,001	1-1,6mm	100	120	3,4

The first variable examined is the contact time at constant temperature. The results of the concentration of reducing sugars at different contact times can be seen in figure 21.

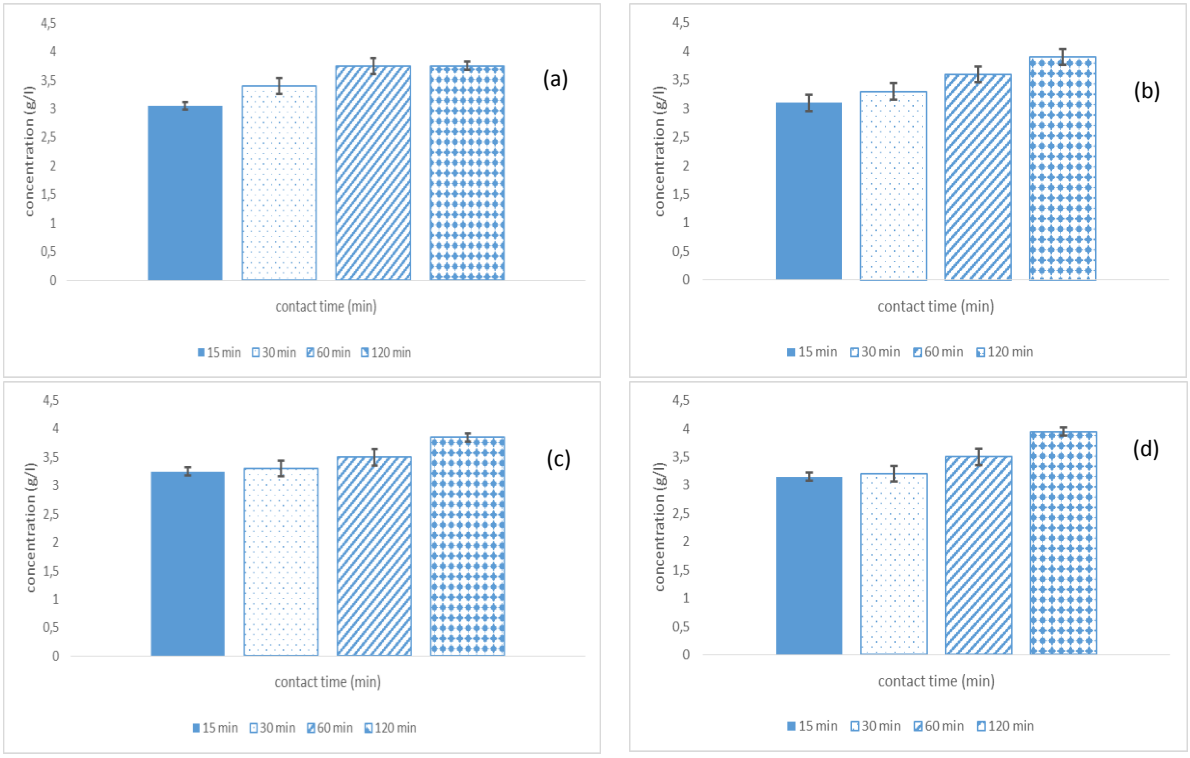
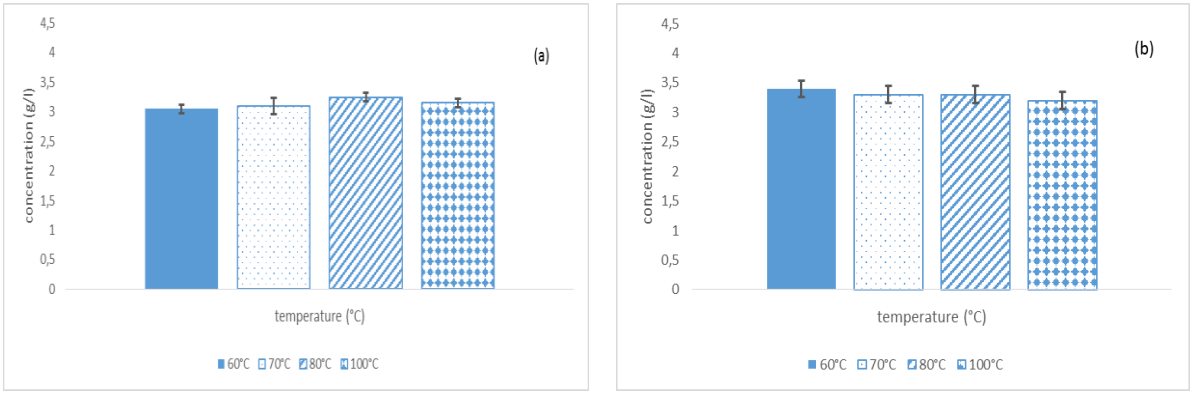


Figure 21: effect of contact time on the concentration of reducing sugars at constant temperature of (a) 60°C, (b) 70°C, (c) 80°C, (d) 100°C.

As can be seen in figure 21, the concentration of reducing sugars rises with longer contact time. This was expected because the solvent has more time to diffuse into the waste and extract the compounds after 120 minutes. It seems that not all reducing sugars have been extracted after 120 minutes.

The second variable examined is the temperature at constant contact time. The concentration of reducing sugars can be seen in figure 22.



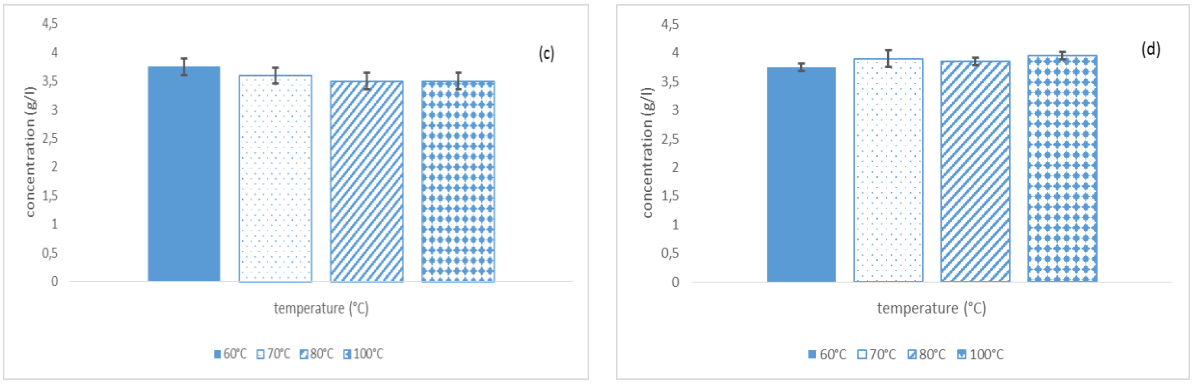


Figure 22: effect of temperature on the concentration of reducing sugars at contact time of (a) 15 minutes, (b) 30 minutes, (c) 60 minutes, (d) 120 minutes.

Temperature doesn't seem to have an effect on the concentration of reducing sugars (see figure 22). The concentration even seems to go down a little at 100°C. It is possible that certain sugars degradate of transform into other compounds at 100°C.

7.3.2.3 Total organic carbon content

The total organic carbon was tested for the first 8 samples of the grape stalk extracts. The results of the total organic carbon, the inorganic carbon and total carbon content can be seen in Table 12.

Table 12: concentration of total carbon, total organic carbon and inorganic carbon in mg/l for grape stalk extracts

	TOC(mg/l)	TC (mg/l)	IC (mg/l)
1a	1859	1863	3,7
1b	1785	1787	2,0
2a	1401	1403	1,7
2b	1307	1310	2,7
3a	1651	1653	2,0
3b	1799	1801	1,9
4a	2206	2210	1,7
4b	2223	2224	1,9
5a	2058	2060	2,0
5b	1983	1985	1,9
6a	2447	2450	2,1
6b	2531	2555	23,7
7a	2860	2862	1,8
7b	2680	2682	2,0
8a	2561	2563	2,2
8b	2907	2909	2,0

The variables examined for the total organic carbon content are ratio solvent/waste, contact time and temperature. The effect of these variables can be seen in figure 23.

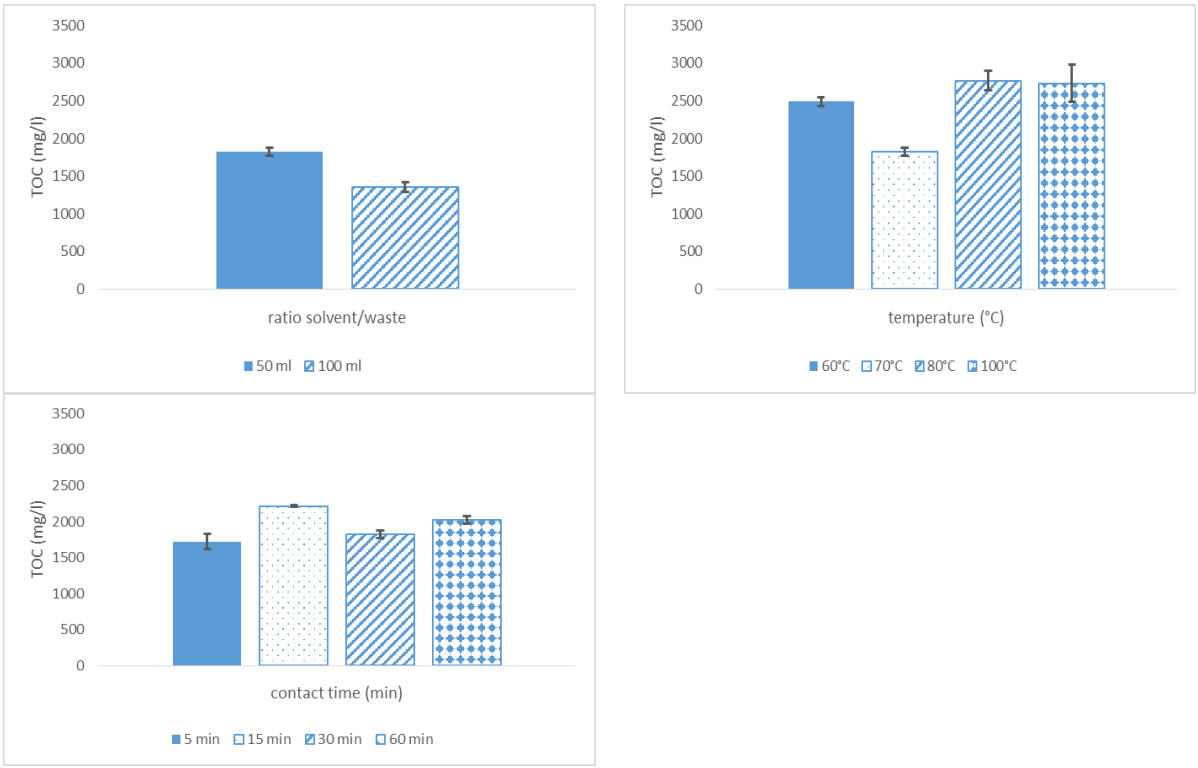


Figure 23: effect of ratio solvent/waste, contact time and temperature on the total organic carbon content

As can be seen in figure 23, there doensn't seem to be a trend in the concentration of total organic carbon. No difinitive conclusion can be drawn concerning the effects of these variables on the TOC content. Only the effect of ratio solvent/waste is clear. The higher the volume, the lower the concentration of organic compounds. This is a normal effect because the same amount of compounds in a lower volume results in a higher concentration (c= n/V).

7.3.2.4 Statistical analysis

A statistical analysis of all the data was performed to determine which parameters could have a significant influence on the concentration of polyphenols and reducing sugars in the extracts obtained. SPSS software program for windows (release 23) was used for the analysis with a significance level of 0,05 (confidence interval 95%) for all the tests.

Temperature and time were the parameters tested. First the mean of the concentration of polyphenols (see table 13) and reducing sugars (see table 14) for each temperature and contact time was calculated.

Box plots were then used to describe the distribution of the data. A box plot contains the median (line across the box), the first (25%) and third (75%) quartiles (bottom and top of the box), lines that extend from bottom and top to the lowest and highest points inside the region (whiskers) and outliers (points outside these limits). The box contains 50% of the data (25-75%). The quartiles divide the data into 4 equal groups with each group comprising a quarter of the data.

Table 13: concentration of polyphenols in the extract obtained at different temperatures and contact time.

	60°C (mg/l)	**70°C (mg/l)**	**80°C (mg/l)**	**100°C (mg/l)**	$\overline{x}$
15 min (mg/l)	542,26 510,06	587,85 525,41	799,84 695,20	867,76 917,56	680,74287
30 min (mg/l)	612,35 519,46	673,26 673,26	758,39 739,06	743,89 821,21	692,61012
60 min (mg/l)	697,21 690,67	714,11 714,11	982,46 937,19	1029,25 1019,19	848,02400
120 min (mg/l)	749,07 763,79	754,35 780,52	901,93 969,05	958,22 1071,68	868,57463
$\overline{x}$	635,60913	677,85750	847,88950	928,59550	

Table 14: concentration of reducing sugars in the extract obtained at different temperatures and contact time.

	60°C (g/l)	**70°C (g/l)**	**80°C (g/l)**	**100°C (g/l)**	$\overline{x}$
15 min (g/l)	3,1 3,0	3,0 3,2	3,2 3,3	3,1 3,2	3,138
30 min (g/l)	3,5 3,3	3,2 3,4	3,4 3,2	3,3 3,1	3,300
60 min (g/l)	3,7 3,8	3,5 3,7	3,4 3,6	3,6 3,4	3,588
120 min (g/l)	3,7 3,8	3,8 4,0	3,9 3,8	3,9 4,0	3,863
$\overline{x}$	3,488	3,475	3,475	3,450	

The boxplot for the concentration of polyphenols at different temperatures can be seen in Figure 24. The concentration of polyphenols at different times can be seen in Figure 25.

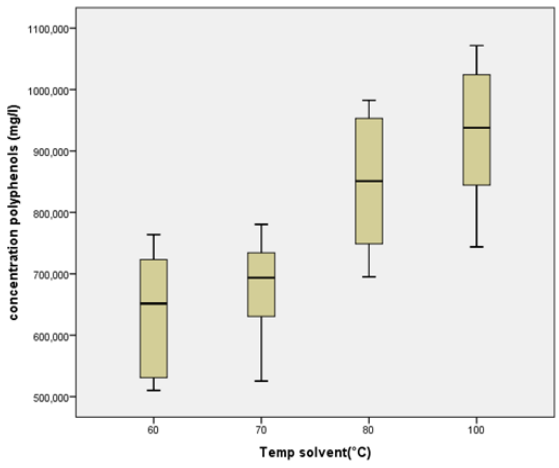


Figure 24: boxplot for the concentration of polyphenols at different temperatures

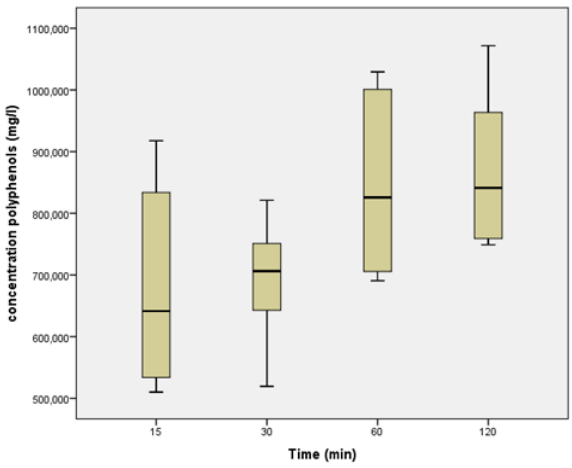


Figure 25: boxplot for the concentration of polyphenols at different contact times

The data points for the concentration of polyphenols at different temperatures (see figure 24) lie in quite equal distance from the median. The medians are relatively equidistant from the top and the bottom of the box and the whiskers are similar in height. There are no outliers present. The box plots at 60-70°C almost have the same values. The same can be said for the box plots at 80-100°C. However, the difference between the 60-70°C group and the 80-100°C group seems important.

The medians for the concentration of polyphenols at different contact times (see figure 25) are not so equidistant from the top and the bottom of the box. This means the data are skewed. But there are also no outliers present. The box plots overlap a little between the different times so there doesn't seem te be a difference.

The box plots for the concentration of reducing sugars at different temperatures can be seen in Figure 26 and the concentration at different times in Figure 27.

For the concentration of reducing sugars at different temperatures (see figure 26), the boxplots are overlapping. There are no outliers present but the medians are not equidistant from the top and the bottom (median is not in the middle of the box and whiskers are not equal). It seems there is no difference between the concentrations of reducing sugars at different temperatures.

The data of the box plots for the concentration of reducing sugars at different contact times (see figure 27) are quite equidistant. The box plots don't overlap much. There is a clear trend in the increase of the concentration of reducing sugars with the increase of extraction temperature.

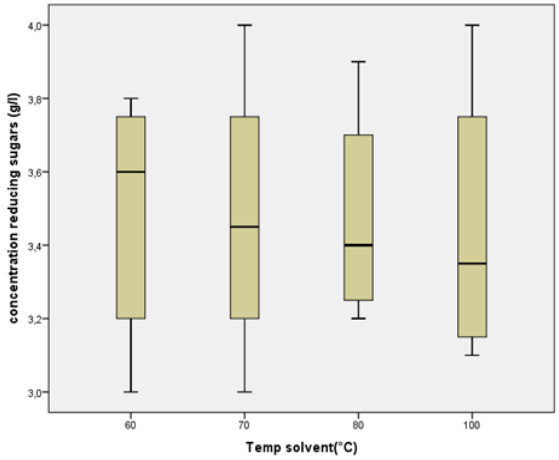


Figure 26: boxplot for the concentrration of reducing sugars at different temperatures

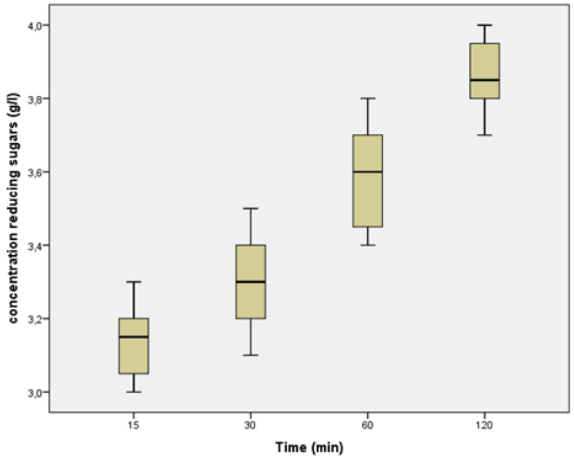


Figure 27: boxplot for the concentration of reducing sugars at different contact times

Following the results of the boxplots, a one-way ANOVA test was done to analyse the differences among group means and their variation. It is a test to see if the means of several groups are equal. If the value is lower than 0,05 (95% confidence level), the differences between the means are significant and further tests are necessary. The results of the ANOVA test for the concentration of polyphenols and reducing sugars at different temperatures can be seen in Table 15. The results of the ANOVA test for the concentration of polyphenols and reducing sugars at different contact times can be seen in Table 16.

Table 15: ANOVA test for the concentration of polyphenols and reducing sugars at different temperatures

		Sum of Squares	df	Mean Square	F	Sig.
concentration polyphenols (mg/l)	Between Groups	461965,566	3	153988,522	14,213	,000
	Within Groups	303359,622	28	10834,272		
	Total	765325,188	31			
concentration reducing sugars (g/l)	Between Groups	,006	3	,002	,019	,996
	Within Groups	2,879	28	,103		
	Total	2,885	31			

Table 16: ANOVA test for the concentration of polyphenols and reducing sugars at different contact times

		Sum of Squares	df	Mean Square	F	Sig.
concentration polyphenols (mg/l)	Between Groups	237887,757	3	79295,919	4,210	,014
	Within Groups	527437,430	28	18837,051		
	Total	765325,188	31			
concentration reducing sugars (g/l)	Between Groups	2,458	3	,819	53,831	,000
	Within Groups	,426	28	,015		
	Total	2,885	31			

The Sig value is lower than 0,05 for the concentration of polyphenols at different temperatures (0,000) and contact times (0,014). For the concentration of the reducing sugars, the value is lower than 0,05 at different contact times (0,000) but not at different temperatures (0,996). The test is considered positive so further analysis is required.

A post hoc Bonferroni test was done to see which variable could be responsible for significant differences between the groups of data. The results of the test for the variable of temperature can be seen in Table 17 and the results for the variable of contact time in Table 18. The test calculates the difference in means between all the variables that need to be compared, the standard deviation and a p-value (sig.) that represents the significance of the difference between the variables tested. If the p-value is lower than 0,05 (confidence interval 95%), the difference is significant.

Table 17: Bonferroni test for temperature

Multiple Comparisons

Bonferroni

Dependent Variable	(I) Temp solvent(°C)	(J) Temp solvent(°C)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
concentration polyphenols (mg/l)	60	70	-42,248375	52,043905	1,000	-189,99752	105,50077
		80	-212,280375*	52,043905	,002	-360,02952	-64,53123
		100	-292,986375*	52,043905	,000	-440,73552	-145,23723
	70	60	42,248375	52,043905	1,000	-105,50077	189,99752
		80	-170,032000*	52,043905	,017	-317,78115	-22,28285
		100	-250,738000*	52,043905	,000	-398,48715	-102,98885
	80	60	212,280375*	52,043905	,002	64,53123	360,02952
		70	170,032000*	52,043905	,017	22,28285	317,78115
		100	-80,706000	52,043905	,793	-228,45515	67,04315
	100	60	292,986375*	52,043905	,000	145,23723	440,73552
		70	250,738000*	52,043905	,000	102,98885	398,48715
		80	80,706000	52,043905	,793	-67,04315	228,45515

*. The mean difference is significant at the 0.05 level.

Table 18: bonferroni test for contact time

Multiple Comparisons

Bonferroni

Dependent Variable	(I) Time (min)	(J) Time (min)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
concentration polyphenols (mg/l)	15	30	-11,867250	68,624068	1,000	-206,68636	182,95186
		60	-167,281125	68,624068	,128	-362,10024	27,53799
		120	-187,831750	68,624068	,064	-382,65086	6,98736
	30	15	11,867250	68,624068	1,000	-182,95186	206,68636
		60	-155,413875	68,624068	,189	-350,23299	39,40524
		120	-175,964500	68,624068	,096	-370,78361	18,85461
	60	15	167,281125	68,624068	,128	-27,53799	362,10024
		30	155,413875	68,624068	,189	-39,40524	350,23299
		120	-20,550625	68,624068	1,000	-215,36974	174,26849
	120	15	187,831750	68,624068	,064	-6,98736	382,65086

		30	175,964500	68,624068	,096	-18,85461	370,78361
		60	20,550625	68,624068	1,000	-174,26849	215,36974
concentration reducing sugars (g/l)	15	30	-,1625	,0617	,082	-,338	,013
		60	-,4500*	,0617	,000	-,625	-,275
		120	-,7250*	,0617	,000	-,900	-,550
	30	15	,1625	,0617	,082	-,013	,338
		60	-,2875*	,0617	,000	-,463	-,112
		120	-,5625*	,0617	,000	-,738	-,387
	60	15	,4500*	,0617	,000	,275	,625
		30	,2875*	,0617	,000	,112	,463
		120	-,2750*	,0617	,001	-,450	-,100
	120	15	,7250*	,0617	,000	,550	,900
		30	,5625*	,0617	,000	,387	,738
		60	,2750*	,0617	,001	,100	,450

*. The mean difference is significant at the 0.05 level.

The bonferroni test for temperature (see table 17) shows that the effect of temperature is significant for the concentration of polyphenols. The p-values for the polyphenols are almost all under 0,05. For the polyphenols the p-values for comparison of 60-70°C and 80-100°C are above 0,05. So the significance lies between the group of 60-70°C and the group of 80-100°C.

For the variable of contact time (see table 18), the p-values are all above 0,05 for the polyphenols and almost all below 0,05 for the reducing sugars. So it seems that contact time doesn't play a significant role for the concentration of polyphenols. It does however seem significant for the concentration of reducing sugars.

7.4 Summary

To obtain the highest concentration of reducing compounds in the preparation of the extracts, temperature and contact time are variables that are important. The concentration of organic compounds (polyphenols and reducing sugars) increases with rising temperature of extraction. The concentration of polyphenols also rises with the increase of contact time. The concentration of reducing sugars does not increase with the increase of contact time. These results were observed when plotting the experimental data and confirmed after statistical analysis. As a conclusion, the highest concentration of reducing agents in the extracts will be obtained at 100°C for 120 minutes for 3 grams in 50 ml Milli-Q.

8 Synthesis and analysis of copper metal nanoparticles

The extracts of spent coffee and grape stalk were put into contact with a synthetic copper solution (copper(II)sulfate, copper(II)chloride). The parameters (temperature, contact time, ratio copper/extract, concentration of metal solution, addition of acid and agitation) that were tested during this contact may affect the formation of copper nanoparticles. The pH of the solution was analysed after 1 hour, 2 days and 4 days of contact time to observe any change resulting from copper nanoparticle formation. A visual observation was performed to determine the effect of the parameters on the presence of fungi and precipitation. UV-Vis spectroscopy and EDX/SEM analysis were performed to determine the presence of copper nanoparticles. A statistical analysis was calculated to determine which parameters had an effect on the presence of fungi and precipitation (see figure 30).

8.1 Materials and Methods

8.1.1 Reagents

Copper(II) sulfate pentahydrate (reagent grade) and copper(II)chloride dihydrate (reagent grade) were bought from Scharlau. Sulfuric acid (95-98%) was bought from ROMIL LTD. Spent coffee extract obtained at 100°C for 120 minutes with particle size 100-250 µm and grape stalk extract obtained at 100°C for 120 minutes with particle size 1-1,6 mm were used for the synthesis.

8.1.2 Analysis of nanoparticles

A Crison pH meter basic 20 was used for the pH measurements. A Shimadzu UVmini-1240 and a UV160A UV-Vis spectrophotometer with Hellma Quartz cuvette were used for the spectra (200-800 nm). A Hitachi model S-4100 field emission SEM (7 kV-WD 5 mm) was used for the particle size analysis. A Zeiss DSM 860A EDX was used for the elemental analysis in which a volume of sample was placed onto a polycarbonate filter (0,22 µm) and kept in the oven overnight at 50°C to dry. After the oven, the filter was coated with a layer of carbon to enhance the conductivity (see figure 28). The samples on the filter with the carbon layer can be seen in figure 29. A visual observation on the presence of fungi and/or precipitation was performed.

Figure 28: instrument for coating of the filter

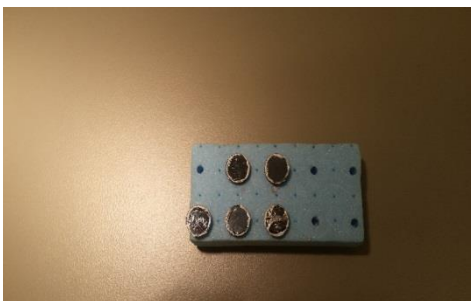


Figure 29: carbon coated samples on polycarbonate filter

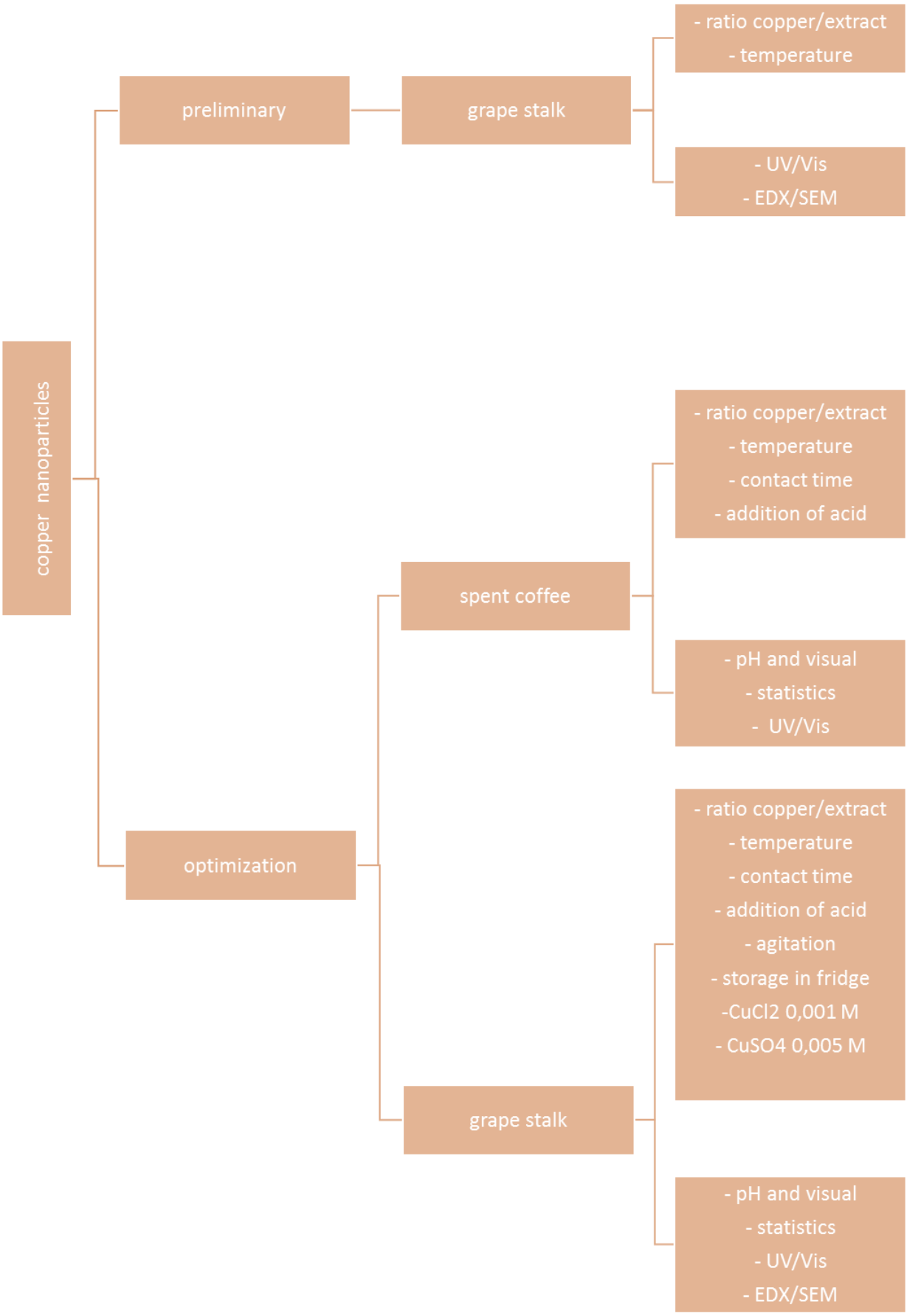


Figure 30: review of all variables examined and analysis performed of copper nanoparticles

8.2 Preliminary research on synthesis of copper metal nanoparticles using grape stalk extract

For the preliminary research on the synthesis of copper metal nanoparticles, several authors were consulted to obtain the information needed regarding the conditions used to obtain the nanoparticles. Grape stalk extracts were selected to make these preliminary assays because they contain a high amount of reducing agents.

8.2.1 Method

Temperature (RT, 60, 90 °C for 1 hour) and ratio copper volume/extract volume (1:5, 2:5, 3:5, 4:5, 5:5, 100:1, 170:30) were the variables examined (see table 19). An aqueous solution of copper(II)sulfate (0,001 M) was used as the source of copper. Extract used for the synthesis, see reagents.

Table 19: synthesis of nanoparticles with parameters ratio copper/solvent and temperature

	Copper solution (ml)	extract (ml)	temperature (°C)
1	1	5	RT
2	2	5	RT
3	3	5	RT
4	4	5	RT
5	5	5	RT
6	100	1	RT
7	170	30	95

The samples were kept in the dark at room temperature and examined on the presence of nanoparticles by UV-Vis absorption spectroscopy and EDX/SEM analysis.

8.2.2 Results and discussion

UV-Vis absorption spectroscopy is used as a technique to analyse the formation of copper nanoparticles because they have the unique characteristic of absorbing strongly in the visible spectrum (Moores & Goettmann, 2006). The surface plasmon band (SPB) is a phenomenon observed due to the presence of nanoparticles, in solution or in the solid phase. Copper nanoparticles (zero-valent, copper(I)oxide, copper(II)oxide) usually absorb between 400-600 nm (Subhankari & Nayak, 2013). Therefore the UV-Vis absorption spectra of the samples were measured and EDX/SEM analysis was used to determine the presence of copper (EDX) and in particular copper nanoparticles (SEM).

8.2.2.1 UV-Vis absorption spectra

UV-Vis absorption spectra were measured for samples 1, 5 and 7. The results of sample 1 can be seen in figure 31, sample 5 in figure 32 and sample 7 in figure 33.

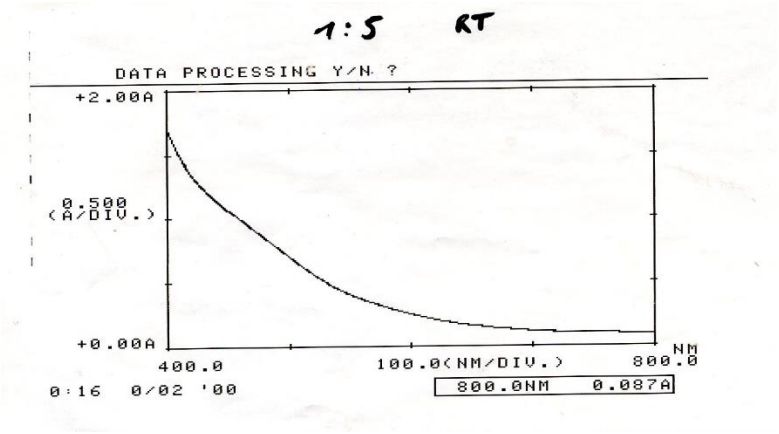


Figure 31: UV-Vis absorption spectra of sample with 1 ml CuSO4 (0,001 M) in 5 ml grape stalk extract at room temperature

As can be seen in figure 31, a beginning of a peak around 450 nm is visible. It is of course partially covered by the larger peak from the organic compounds starting at 400 nm. This means that there is a possibility that copper nanoparticles have been synthesized.

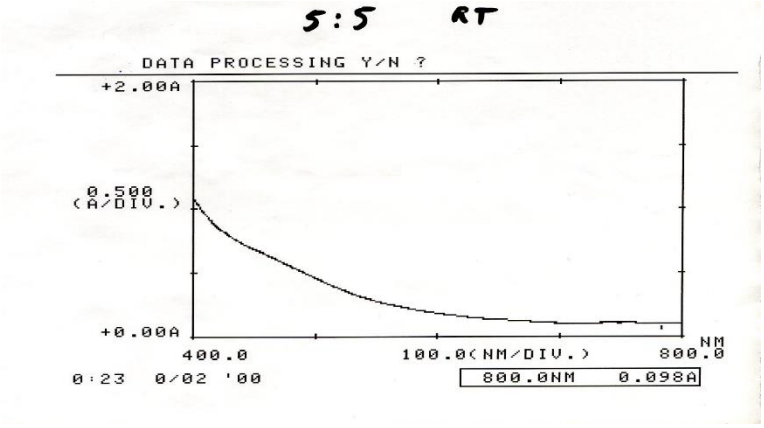


Figure 32: UV-Vis absorption spectra of sample with 5 ml CuSO4 0,001 M in 5 ml grape stalk extract at room temperature

In figure 32, the peak around 450 nm seems smaller than the previous sample. This could be a result of the higher total volume (same amount particles in higher volume results in lower concentration). This also means that the amount of nanoparticles synthesized does not seem to increase with higher volume of copper solution added.

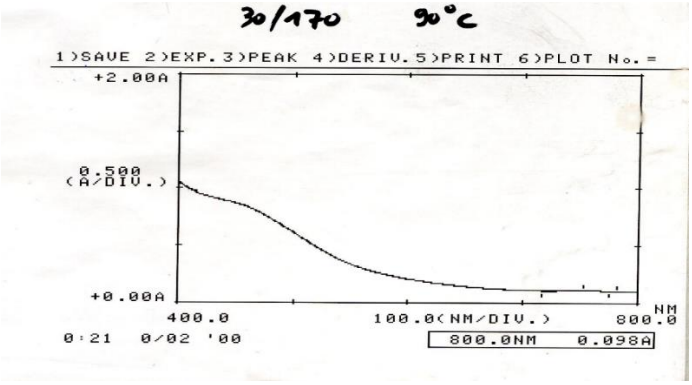


Figure 33: UV-Vis absorption spectra of sample with 30 ml of grape stalk extract and 170 ml of CuSO4 0,001 M at 90°C for 1 hour

As can be seen in figure 33, at 90°C the peak between 400-450 nm is more pronounced. It must be pointed out that a red color change was observed after 1 hour. This was not the case with the previous samples at room temperature. This change could be an indicator that copper(I)oxide nanoparticles are formed because the solid

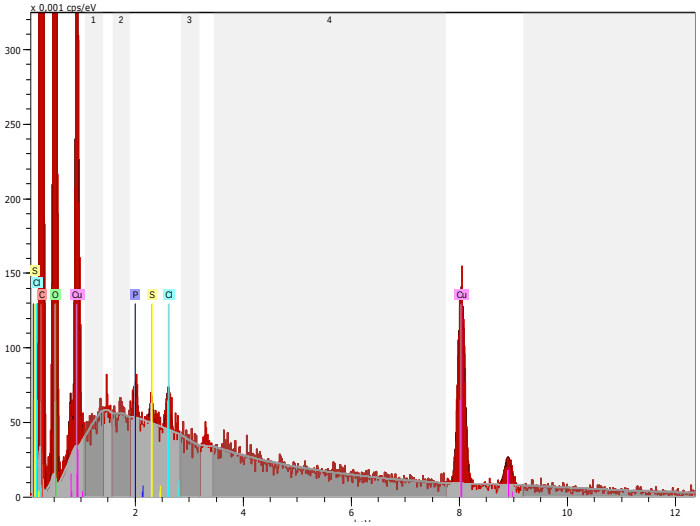
of this oxide has a red color. Contrary to this observation, only a green color change due to formation of copper zero-valent metal is mentioned in literature (Subhankari & Nayak, 2013). In general, it seems that the temperature is a variable that could have an influence on the synthesis of copper nanoparticles.

8.2.2.2 EDX/SEM

Energy dispersive x-ray spectroscopy was performed to determine the elemental composition of the samples. More specifically to see if copper is present.

EDX analysis was performed on preliminary samples 1:100 and 30:170. Sample 30:170 was synthesized twice. The first sample was measured a couple of days (30/170 (1)) and the second a couple of hours after synthesis (30/170 (2)).

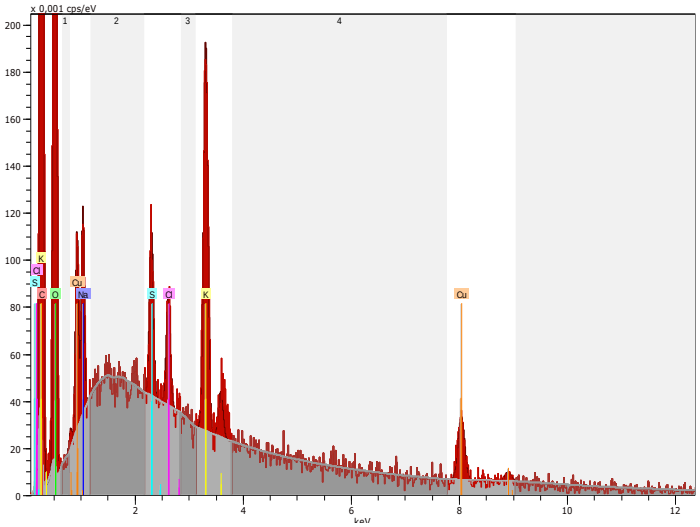
The result of the EDX analysis of sample 1:100 can be seen in figure 34 and the results of sample 30:170 (1) and (2) can be seen in figure 35 and 36.



Spectrum: 8B 1/100 2

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]
Carbono	K-series	54,24	54,24	64,13
Oxígeno	K-series	38,49	38,49	34,16
Fósforo	K-series	0,13	0,13	0,06
Cloro	K-series	0,17	0,17	0,07
Cobre	K-series	6,88	6,88	1,54
Azufre	K-series	0,08	0,08	0,04
Total:		100,00	100,00	100,00

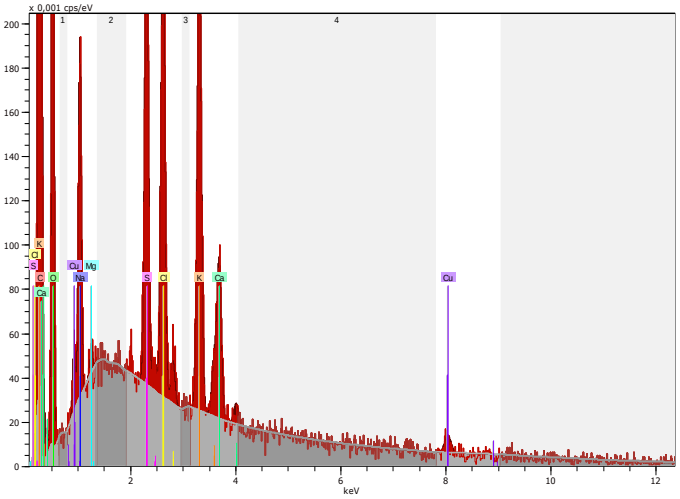
Figure 34: EDX report of sample 1/100 (1 ml extract in 100 ml copper solution)



Spectrum: 30/170 (1) 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]
Carbono	K-series	55,38	55,38	64,05
Oxígeno	K-series	39,08	39,08	33,94
Sodio	K-series	0,99	0,99	0,60
Azufre	K-series	0,47	0,47	0,20
Cloro	K-series	0,35	0,35	0,14
Potasio	K-series	1,89	1,89	0,67
Cobre	K-series	1,84	1,84	0,40
Total:		100,00	100,00	100,00

Figure 35: EDX report of sample 30/170 (1) (30 ml grape stalk in 170 ml copper solution)



Spectrum: 30/170 (2) 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom.C [at.%]
Carbono	K-series	69,97	69,97	78,80
Oxígeno	K-series	20,42	20,42	17,26
Sodio	K-series	1,88	1,88	1,11
Magnesio	K-series	0,10	0,10	0,06
Azufre	K-series	1,71	1,71	0,72
Cloro	K-series	2,08	2,08	0,79
Potasio	K-series	2,35	2,35	0,81
Calcio	K-series	1,05	1,05	0,35
Cobre	K-series	0,46	0,46	0,10
	Total:	100,00	100,00	100,00

Figure 36: EDX report of sample 30/170 (2)

For the preliminary samples (1/100 and 30/170), the EDX reports show the presence of copper (element)(see figure 34-36). This means that there is a possibility that copper nanoparticles are present. The concentration of carbon in the samples is high. This is because the extract contains a lot of organic compounds which could also be seen in the UV-Vis absorption spectra (peak between 300-400 nm).

SEM analysis was performed to observe the synthesized nanoparticles. SEM analysis on preliminary samples 6 (1/100) and 7 (30/170) was performed.

The results of the SEM analysis of sample 1/100 can be seen in figure 37 and 38.

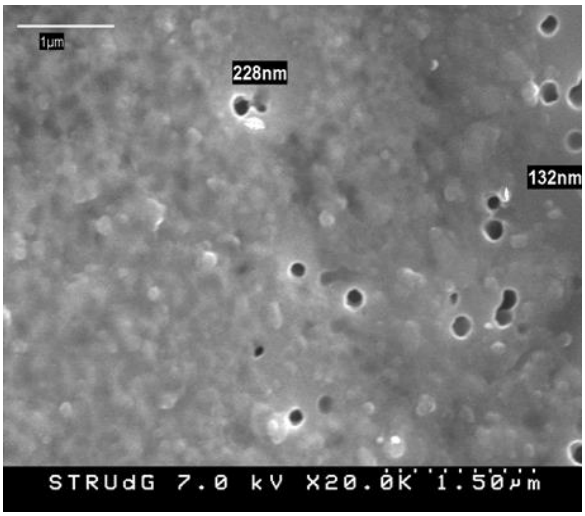


Figure 37: sample 1:100 diluted (1:2) at 20 000x magnification

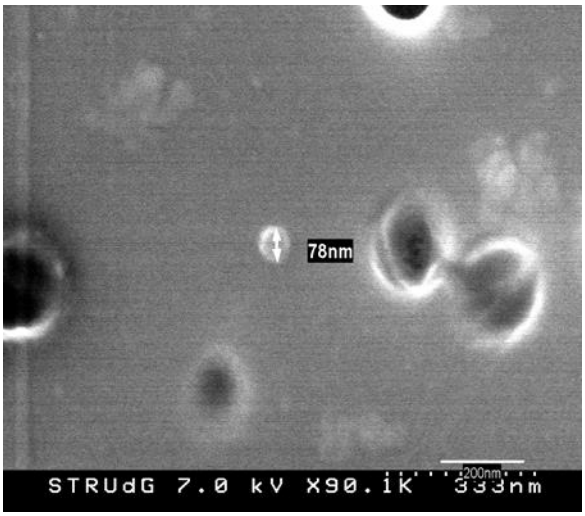


Figure 38: sample 1:100 diluted (1:2) at 90 000x magnification

As can be seen in figure 37, nanoparticles seem to be present. There are however a lot of particles which makes it difficult to distinguish between them and isolate single particles for size analysis. The black holes are the pores of the polycarbonate filter. One part of the filter is further magnified to try and find an isolated particle (see figure 38). The higher the amount of particles, the more chance they form aggregates. This means that it is possible that there are even smaller particles present than cannot be seen in these figures because they are obscured by the vast amount of particles.

The results of the SEM analysis of sample 30/170 (1) and (2) can be seen in figure 39 and 40.

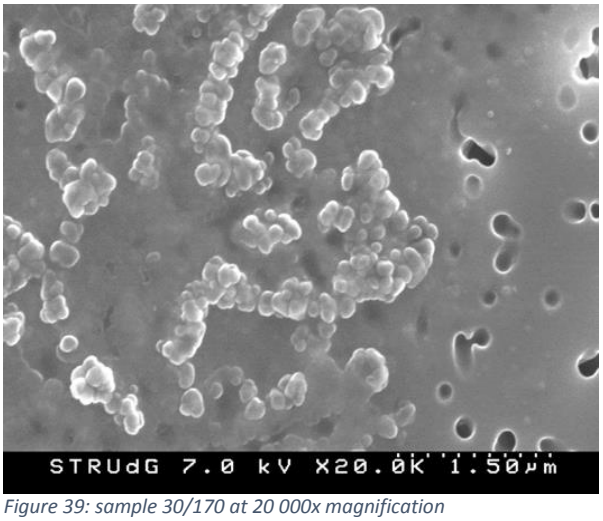


Figure 39: sample 30/170 at 20 000x magnification

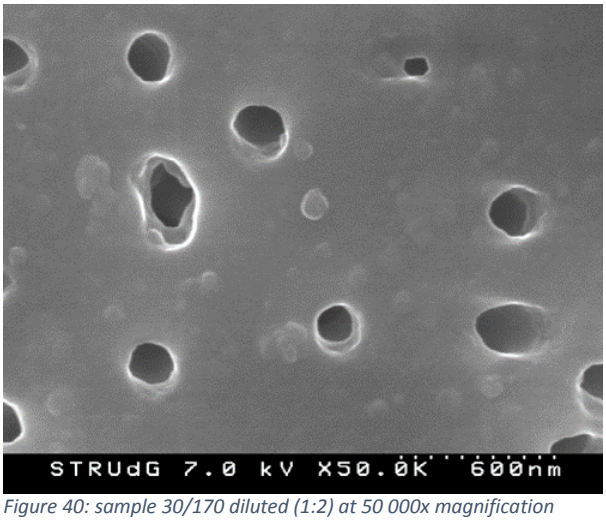


Figure 40: sample 30/170 diluted (1:2) at 50 000x magnification

As can be seen in figure 39, nanoparticles are again present. They seem to form long chains and aggregates. This can be explained by the fact that there are again a lot of particles present which promotes the formation of aggregates. The sample was diluted and a larger magnification was used to try and find isolated particles (see figure 40).

Supplementary images can be found in Annex I.

8.3 Synthesis of copper metal nanoparticles

After the preliminary research of the synthesis of copper nanoparticles using grape stalk, parameters of contact time, addition of acid (16 µl H_2SO_4 16%), agitation, storage in the fridge, use of copper(II)chloride and copper concentration were added to optimize the synthesis of copper nanoparticles. The synthesis were performed using spent coffee and grape stalk extracts.

8.3.1 Synthesis of copper nanoparticles using spent coffee extract

An aqueous solution of copper(II)sulfate (0,001 M) was used as the source of copper. The variables that were examined are ratio copper/extract (1, 2, 4, 6 ml copper solution), temperature (room temp or RT, 60°C and 90°C), contact time (1 hour, 2 days and 4 days) and addition of 16 µl of sulfuric acid 16% (presence of acid (+), absence of acid (-)). A summary of these conditions can be seen in table 20.

Table 20: conditions for synthesis of copper nanoparticles using spent coffee extract

	copper solution (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)
1	1	10	RT	-
2	2	10	RT	-
3	4	10	RT	-
4	6	10	RT	-
5	1	10	60	-
6	2	10	60	-
7	4	10	60	-
8	6	10	60	-
9	1	10	90	-

10	2	10	90	-
11	4	10	90	-
12	6	10	90	-
13	1	10	RT	+
14	2	10	RT	+
15	4	10	RT	+
16	6	10	RT	+
17	1	10	60	+
18	2	10	60	+
19	4	10	60	+
20	6	10	60	+
21	1	10	90	+
22	2	10	90	+
23	4	10	90	+
24	6	10	90	+

After synthesis, the samples were kept at room temperature in the dark for analysis.

The results obtained by UV-Vis absorption spectroscopy revealed that the amount of copper nanoparticles formed was really low when using coffee extract (see section 8.3.4.1.2). Therefore, the decision was made to proceed with the use of grape stalk extract (higher concentration of reducing agents).

8.3.2 Synthesis of copper nanoparticles using grape stalk extract

Samples with ratio copper volume/extract volume of 1:10, 2:10, 3:10, 4:10, 5:10 and 6:10 were prepared at different temperatures (room temp, 60, 90°C) with (+) or without (-) the addition of acid (16 µl H_2SO_4 16%). An aqueous solution of copper(II)sulfate (0,001 M) was used as the source of copper.

Table 21: synthesis of copper nanoparticles with parameters ratio copper/extract, temperature and pH

	Copper solution (ml)	**extract (ml)**	**temperature (°C)**	**16 µl H_2SO_4 (16%)**
1	1	10	RT	-
2	2	10	RT	-
3	3	10	RT	-
4	4	10	RT	-
5	5	10	RT	-
6	6	10	RT	-
7	1	10	60	-
8	2	10	60	-
9	4	10	60	-
10	6	10	60	-
11	1	10	RT	+
12	2	10	RT	+
13	4	10	RT	+
14	6	10	RT	+
15	1	10	90	-

16	2	10	90	-
17	4	10	90	-
18	6	10	90	-
19	1	10	60	+
20	2	10	60	+
21	4	10	60	+
22	6	10	60	+
23	1	10	90	+
24	2	10	90	+
25	4	10	90	+
26	6	10	90	+

Samples 1-26 were kept in the dark at room temperature for further analysis.

A new set of samples was made using the same conditions as samples 1-26 but with the addition of agitation as a new variable (table not shown).

The next variable examined was the effect of keeping the samples stored in the fridge on the visual observation (presence of fungi and/or precipitation). The conditions of the samples can be seen in table 22.

Table 22: conditions for samples stored in the fridge after synthesis

	Copper solution (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)
27	2	10	60	-
28	4	10	60	-
29	6	10	60	-
30	2	10	90	-
31	4	10	90	-
32	6	10	90	-
33	2	10	RT	-
34	4	10	RT	-
35	6	10	RT	-
36	4	10	RT	+
37	4	10	60	+
38	4	10	90	+

The next variable examined was the use of an aqueous solution of copper(II)chloride (0,001 M) instead of copper(II)sulfate (0,001 M). The conditions of the samples can be seen in table 23.

Table 23: conditions for synthesis with copper(II)chloride 0,001 M

	Copper solution (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)
39	2	10	90	-
40	4	10	90	-
41	6	10	90	-
42	4	10	90	+
43	2	10	60	-

44	4	10	60	-
45	6	10	60	-
46	4	10	60	+

The last variable examined was the concentration of the aqueous copper metal solution. An aqueous solution of copper(II)sulfate (0,005 M) was used. Table 24 shows the conditions used.

Table 24: conditions for synthesis with copper(II)sulfate 0,005 M

	Copper solution (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)
47	4	10	RT	-
48	4	10	60	-
49	4	10	90	-
50	4	10	60	+
51	2	10	RT	-
52	2	10	60	-
53	2	10	90	-
54	2	10	60	+

8.3.3 Analysis of the copper nanoparticles

A visual oberservation of the samples (coffee and grape stalk) was performed after 4 days to determine if fungi and/or precipitation was present. The statistical analysis program SPSS (version 23) for windows was used to determine which parameters (ratio CuSO4/extract volume, temperature, addition of acid, agitation and preservation of the samples (fridge or RT)) could have an influence on the presence of fungi and/or precipitation.

The pH and UV-Vis spectra (200-800 nm) of samples 1-24 from spent coffee and samples 1-54 from grape stalk were measured after 1 hour, 2 days and 4 days . EDX and SEM analysis were performed on selected samples 11 (without agitation), 19 (with and without agitation) and 23 (without agitation) from grape stalk.

For the EDX and SEM analysis of the samples, 2 methods were used. For the first method, 3 ml of the sample was put on the filter, dried in the oven (50°C) overnight and coated with carbon. For the second method, 3 ml of sample was centrifuged (20 000 rpm for 10 minutes). Then the pellet was washed and redispersed with Milli-Q. Then 1 ml of sample was put on the filter, dried in the oven and coated with carbon.

8.3.4 Results and discussion

8.3.4.1 Results using spent coffee extract

For the results of the synthesis of copper nanoparticles using spent coffee extract, 4 analysis were used. First the pH was measured after 1 hour, 2 days and 4 days. After 4 days the samples were examined for the presence of fungi and/or precipitation. UV-Vis absorption spectra (200-800 nm) of the samples were measured after 1 hour, 2 days and 4 days.

8.3.4.1.1 pH and visual observation

The results of the pH after 1 hour, 2 days and 4 days as well as the visual observation of the samples after 4 days can be seen in Table 25. The volume of spent coffee extract is constant (10 ml). The variables examined in this table are ratio CuSO4/extract volume, temperature, addition of acid and contact time.

Table 25: effect of variables ratio copper/extract, temperature, addition of acid and contact time on pH and presence of fungi and/or precipitation

	copper solution (ml)	extract (ml)	temperature (°C)	H_2SO_4 (16%)	pH after 1H	pH after 2 days	pH after 4 days	Fungi after 4 days	Precipitation after 4 days
1	1	10	RT	-	3,83	3,78	3,78	+	-
2	2	10	RT	-	3,75	3,76	3,76	-	+
3	4	10	RT	-	3,69	3,75	3,73	-	-
4	6	10	RT	-	3,72	3,74	3,72	-	-
5	1	10	60	-	3,69	3,66	3,78	-	-
6	2	10	60	-	3,65	3,70	3,75	-	-
7	4	10	60	-	3,64	3,67	3,72	-	+
8	6	10	60	-	3,69	3,70	3,76	-	+
9	1	10	90	-	3,73	3,77	3,80	-	-
10	2	10	90	-	3,68	3,75	3,74	-	+
11	4	10	90	-	3,67	3,72	3,71	-	+
12	6	10	90	-	3,66	3,71	3,70	-	+
13	1	10	RT	+	2,45	2,49	2,48	-	+
14	2	10	RT	+	2,33	2,35	2,35	-	+
15	4	10	RT	+	2,73	2,76	2,76	-	+
16	6	10	RT	+	2,10	2,14	2,17	-	-
17	1	10	60	+	2,36	2,47	2,46	-	+
18	2	10	60	+	2,24	2,36	2,36	-	+
19	4	10	60	+	2,41	2,52	2,52	-	+
20	6	10	60	+	2,39	2,51	2,53	-	+
21	1	10	90	+	2,52	2,58	2,58	-	+
22	2	10	90	+	2,64	2,70	2,72	-	+
23	4	10	90	+	2,62	2,70	2,70	-	+
24	6	10	90	+	2,60	2,67	2,66	-	+

For the pH, the effect of ratio CuSO4/extract volume on the pH can be seen between samples 1, 2, 3 and 4 (see table 25). It seems the higher the ratio (more CuSO4), the lower the pH untill a ratio of 3:5 (sample 4) is reached. Then the pH rises again.

The effect of temperature on the pH can be seen between samples 2, 6 and 10 (see table 25). The pH seems to drop between room temperature (sample 2) and 60°C (sample 6). It rises again between 60°C (sample 6) and 90°C (sample 10).

The effect of contact time on the pH can be seen in sample 6. The pH rises between contact times of 1 hour, 2 days and 4 days. This is not the case for all samples. Sometimes the pH drops a little between 2 days and 4 days. But in general, there is a rising trend in pH.

For the visual observation (fungi, precipitation), the effects of ratio CuSO4/extract volume, temperature, addition of acid and contact time are clear when it comes to the presence of fungi (none present). In general the variables don't seem to have an effect on the presence of fungi.

The temperature seems to have an effect on the presence of precipitation. The rise in temperature results in more samples with precipitation. This is to be expected because the particles formed have a higher kinetic energy with higher temperatures. They are more likely to come into contact with each other to form aggregates. The addition of one drop of acid (H_2SO_4 16%) also seems to promote the precipitation.

8.3.4.1.2 Statistical analysis

A statistical analysis was performed to see which parameters (ratio copper/extract, temperature, addition of acid) could have an effect on the presence of fungi and/or precipitation. SPSS software program for windows (release 23) was used fort he analysis with a significance level of 0,05 (confidence interval 95%) for all the tests.

First a crosstab analysis of the data of observation (fungi or precipitation) and each of the variables was carried out. These crosstabs give an idea if the connection between the observation and the variable is symmetrical or asymmetrical. In a cross-tab you can see exactly how many times each combination of variable and observation (positive or negative) occurs. The cells of the table indicate the number of times the combination occurs again. If the actual count and the expected count are very different, the effect of the variable on the observation could be important.

To test whether there is a correlation between the variable and the observation a chi-square test can be performed. If the Pearson Chi-Square significance value is lower than 0,05, the test is considered positive and the variable has an influence on the observation. If more than a quarter of the cells have an expected frequency of 5 or less, the results of the test can't be interpreted.

Only the results of combination of variables and observations that have a significance value lower than 0,05 are shown here. The results of the other tests can be found in Annex II.

The crosstab and subsequent Chi-Square test of variable addition of acid on the presence of precipitation can be seen in table 26 and 27.

Table 26: crosstab for variable addition of acid on presence of precipitation

Crosstab

			H2SO4 (16%)		
			-	+	Total
precipitation	-	Count	6	1	7
		Expected Count	3,5	3,5	7,0
		% of Total	25,0%	4,2%	29,2%
	+	Count	6	11	17
		Expected Count	8,5	8,5	17,0
		% of Total	25,0%	45,8%	70,8%
Total		Count	12	12	24
		Expected Count	12,0	12,0	24,0
		% of Total	50,0%	50,0%	100,0%

As can be seen in table 26, the expected number of samples with or without the presence of precipitation and the actual number of samples with or without the presence of precipitation are shown. If the difference between the counts is high, for example count 6 and expected count 3,5 for precipitation – and acid -, the possibility of a correlation between the observation and the variable is high.

Table 27: Chi-Square test of variable addition of acid on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5,042[a]	1	,025		
Continuity Correction[b]	3,227	1	,072		
Likelihood Ratio	5,455	1	,020		
Fisher's Exact Test				,069	,034
N of Valid Cases	24				

a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 3,50.

b. Computed only for a 2x2 table

The Pearson Chi-Square significance value is 0,025. The addition of acid seems to have a positive effect on the presence of precipitation (more samples positive with acid). This was unexpected because a low pH (<5,5) usually prevents metals from precipitating.

Because the preliminary results showed that there is more chance of producing copper nanoparticles with grape stalk extract, the decision was made to proceed with grape stalk extract and try to optimize the preliminary results.

8.3.4.1.3 UV-Vis absorption spectra

The UV-Vis spectra of the samples were measured after 1 hour, 2 days and 4 days (contact time). A 0,001 M CuSO4 solution was used as the source of copper. The variables examined with this analysis are ratio CuSO4/extract volume, temperature, addition of acid and contact time. Selected spectra were used to determine the effect of the variables.

Spectra of samples with 1, 2, 4 and 6 ml of copper solution in 10 ml of extract at room temperature taken after 1 hour show the effect of the ratio copper/extract volume in figure 41.

As can be seen in figure 41, the ratio of CuSO4/extract volume only seems to have an effect on the concentration of organic compounds (peak between 300-400 nm). This is to be expected because the total volume rises with the amount of CuSO4 added. The same amount of organic compounds in a higher volume results in a lower concentration which in turn results in a lower peak in the spectrum. No peak is visible around 500 nm. This means that either no copper nanoparticles were formed or that the concentration of copper nanoparticles is too low to be detect.

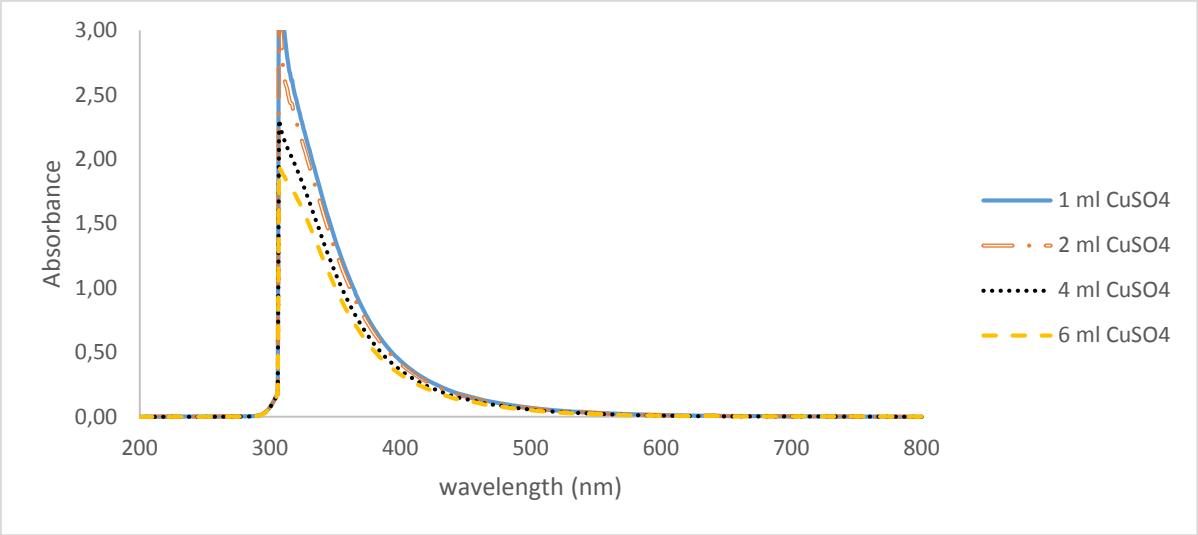


Figure 41: effect of ratio copper/extract volume on UV-Vis absorption spectra at room temperature after 1 hour without acid and with 10 ml of spent coffee extract

The next variable examined was the temperature (RT, 60 °C, 90°C). The UV-Vis absorption spectra at different temperatures can be seen in figure 43. The extract volume (10 ml), contact time (1 hour), ratio CuSO4/extract volume (4:10) and addition of acid (no acid) were kept constant.

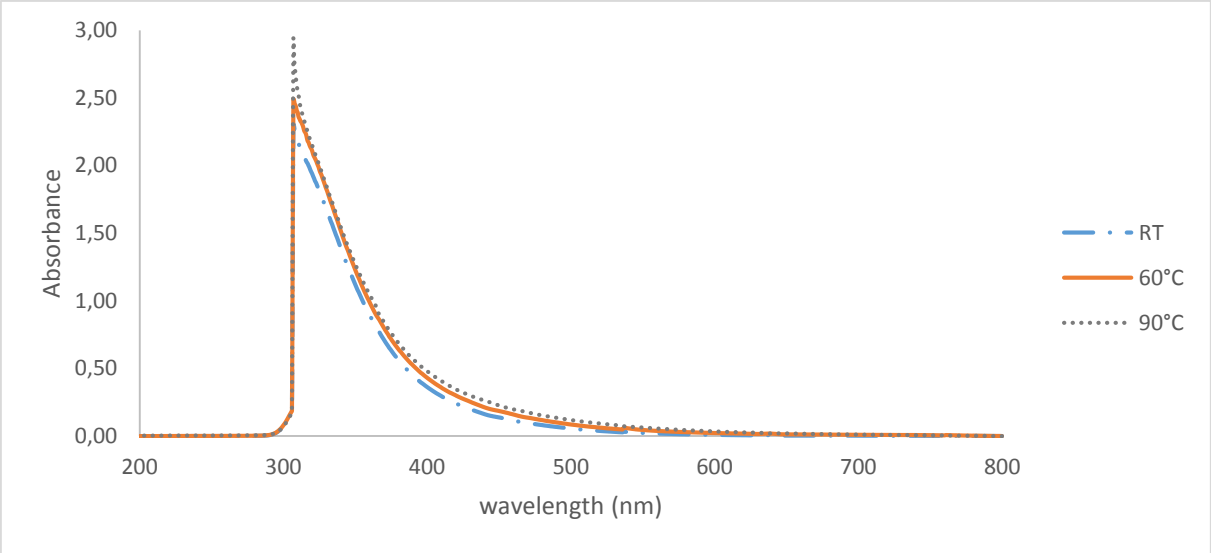


Figure 43: effect of temperature on UV-Vis absorption spectra for 4 ml of CuSO4 after 1 hour without acid and with 10 ml of spent coffee extract

As can be seen in figure 43, the absorbance of the whole spectrum seems to rise a little with rising temperature. There is again no peak visible around 500 nm. The concentration of nanoparticles formed is probably too low to be detected due to the width of the absorbance band of the organic compounds. The effect of the addition of acid was the next variable examined. The UV-Vis absorption spectra with or without acid can be seen in figure 44. The extract volume (10 ml), contact time (1 hour), ratio CuSO4/extract volume (4:10) and temperature (RT) were kept constant.

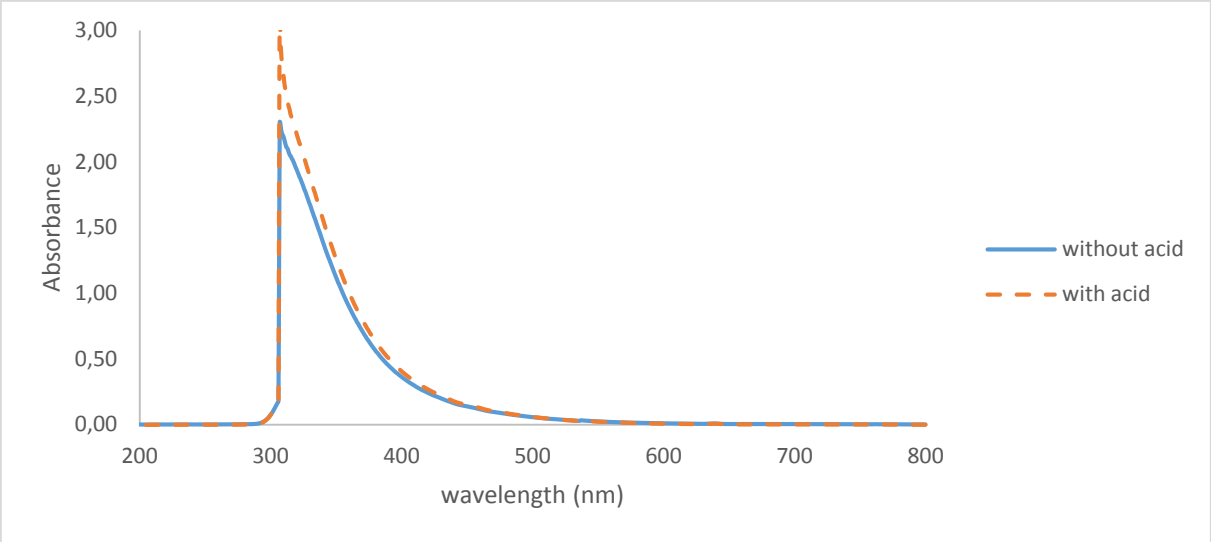


Figure 44: effect of addition of 1 drop H2SO4 (16%) on the UV-Vis absorption spectra of 4 ml CuSO4 at RT after 1 hour with 10 ml of spent coffee extract

As can be seen in figure 44, the addition of acid only has an effect on the peak resulting from the organic compounds in the extract. The concentration of organic compounds seems to be higher with the addition of acid. Again, no peak can be seen around 500 nm.

The last variable examined is the contact time (1 hour, 2 days, 4 days). The result can be seen in figure 45. The extract volume (10 ml), addition of acid (no acid), ratio CuSO4/extract volume (6:10) and temperature (90°C) were kept constant.

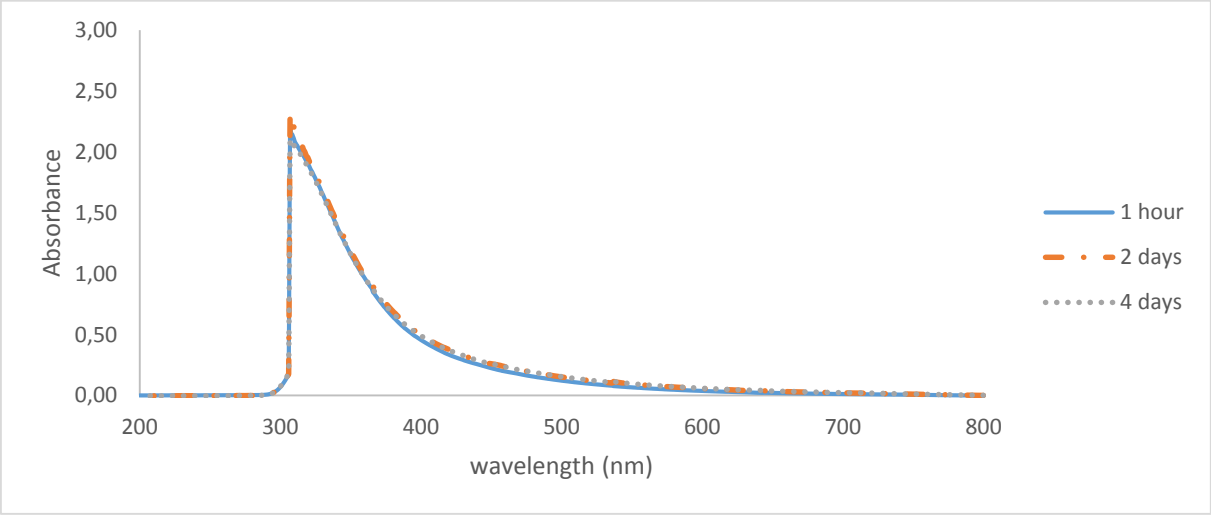


Figure 45: effect of contact time on the UV-Vis absorption spectra of 6 ml CuSO4 at 90°C without acid and with 10 ml of coffee extract

As can be seen in figure 45, the contact time doesn't seem to have an effect on the absortion of the samples. It seems that nothing happens concerning the formation of copper nanoparticles over time.

8.3.4.2 Results using grape stalk extracts

The pH of the solution was measured after 1 hour, 2 days and 4 days (contact time). After 4 days the samples were checked for the presence of fungi and/or precipitation. UV-Vis absorption spectra (200-800 nm) were measured after 1 hour, 2 days and 4 days. EDX/SEM analysis was performed on selected samples to determine

the presence of copper nanoparticles and a statistical analysis was calculated to determine which parameters (temperature, ratio copper/extract, addition of acid, samples kept in fridge and agitation) had an effect on the visual observations.

8.3.4.2.1 pH and visual observation

The effect of ratio copper/extract, temperature, contact time and addition of H_2SO_4 can be seen in table 28.

Table 28: effect of ratio copper/sovent, temperature, contact time and addition of acid on pH and visual observations

	$CuSO_4$ (ml)	extract (ml)	temp (°C)	16 µl H_2SO_4 (16%)	pH after 1h	pH after 2 days	pH after 4 days	Fungi after 4 days	Precipitation after 4 days
1	1	10	RT	-	4,23	4,23	4,20	+	+
2	2	10	RT	-	4,21	4,21	4,22	+	+
3	3	10	RT	-	4,21	4,20	4,23	+	+
4	4	10	RT	-	4,21	4,21	4,22	+	+
5	5	10	RT	-	4,21	4,20	4,22	+	+
6	6	10	RT	-	4,20	4,19	4,24	+	+
7	1	10	60	-	4,05	4,21	4,24	+	-
8	2	10	60	-	4,04	4,21	4,22	-	-
9	4	10	60	-	4,06	4,20	4,22	-	-
10	6	10	60	-	4,08	4,22	4,20	-	-
11	1	10	RT	+	3,44	3,45	3,44	-	+
12	2	10	RT	+	3,28	3,26	3,29	-	+
13	4	10	RT	+	3,45	3,44	3,43	-	+
14	6	10	RT	+	3,01	2,99	2,99	-	+
15	1	10	90	-	4,15	4,15	4,19	+	-
16	2	10	90	-	4,15	4,19	4,18	+	-
17	4	10	90	-	4,15	4,19	4,20	+	-
18	6	10	90	-	4,14	4,18	4,19	+	-
19	1	10	60	+	3,66	3,65	3,67	+	-
20	2	10	60	+	3,65	3,59	3,59	+	-
21	4	10	60	+	3,27	3,36	3,38	+	-
22	6	10	60	+	3,02	3,86	3,87	+	-
23	1	10	90	+	3,57	3,70	3,71	+	+
24	2	10	90	+	3,51	3,68	3,69	+	+
25	4	10	90	+	3,28	3,30	3,31	+	-
26	6	10	90	+	3,77	3,07	3,07	+	-

A change in color of the solution to red was observed with higher temperatures (60, 90°C) (see figure 46). This could mean that copper(I)oxide nanoparticles are present in the solution.

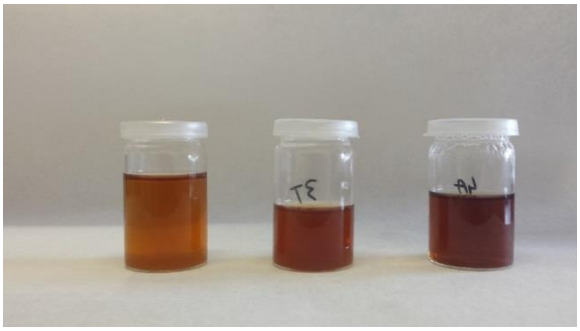


Figure 46: color change to red after synthesis at higher temperatures (RT, 60°C, 90°C)

As can be seen in table 28, for contact time, the pH at room temperature doesn't change at different contact times (with or without acid). At 60°C (without acid) and 90°C (without acid) the pH rises between 1 hour and 2 days. It remains constant between 2 days and 4 days. At 60°C with acid, no difinitive conclusion can be made. However, it seems that with a higher amount of copper, the pH rises. At 90°C with acid, no conclusion about the pH can be made.The ratio of copper/extract volume doesn't seem to have an effect on the pH. For the temperature, the pH drops between room temperature and 60°C but rises again between 60°C and 90°C.

For the visual observations (see table 28), the temperature seems to have an effect on both the presence of fungi and the presence of precipitation. No precipitation is observed when the temperature increases. Concerning the presence of fungi, 60°C exhibits the best result (no fungi) but with 90°C fungi are again present.

The ratio copper/extract volume doesn't have an effect on the visual observations. Fungi and/or precipitation are either present or not. This was not expected. Copper is supposed to have anti-bacterial properties. But it is possible that the concentration of copper solution is too low to have this effect.

The addition of acid seems to promote fungi growth but decreases the chance of precipitation with rising temperatures (60°C, 90°C). This was partially expected and unexpected. The addition of acid usually slows down the bacterial growth. A low pH also prevents the precipitation of metals (<5,5).

Samples 1-26 were repeated in a new set with the addition of agitation. The visual observations are not shown in a table because they were all positive for these samples. It seems agitation promotes both the growth of fungi and the formation of precipitation.

Another variable tested was the effect of keeping the samples stored in the fridge instead of room temperature. The visual observations for this variable can be seen in table 29.

Table 29: effect of keeping samples in the fridge on the presence of fungi and precipitation

	$CuSO_4$ (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)	fungi	precipitation
27	2	10	60	-	-	-
28	4	10	60	-	-	-
29	6	10	60	-	-	-
30	2	10	90	-	-	-
31	4	10	90	-	-	-
32	6	10	90	-	-	-
33	2	10	RT	-	-	+
34	4	10	RT	-	-	+
35	6	10	RT	-	-	-
36	4	10	RT	+	-	+
37	4	10	60	+	-	+
38	4	10	90	+	+	+

As can be seen in table 29, the lower temperature of the fridge slows down the growth of fungi. This is to be expected because the cold slows down the metabolism of the fungi present. It does not seem to have a difinitive effect on the presence of precipitation.

The next variable examined is the use of copper(II)chloride instead of copper(II)sulfate. The effect of this variable on the visual observations can be seen in table 30.

Table 30: effect of using CuCl2 0,001 M solution instead of CuSO4 0,001 M on the presence of fungi and precipitation

	$CuCl_2$ (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)	fungi	precipitation
39	2	10	90	-	+	-
40	4	10	90	-	+	-
41	6	10	90	-	+	+
42	4	10	90	+	+	+
43	2	10	60	-	+	-
44	4	10	60	-	+	-
45	6	10	60	-	+	-
46	4	10	60	+	+	-

As can be seen in table 30, fungi are present in every sample. It seems that $CuSO_4$ has a kind of antibacterial property that is different from $CuCl_2$. In the case of precipitation, it seems to slow down the formation of aggregates in general.

The last variable examined is a higher concentration of copper (0,005 M). The results for the visual observation of this variable can be seen in table 31.

Table 31: effect of higher copper concentration (0,005 M) on presence of fungi and precipitation

	CuSO4 (ml)	extract (ml)	temperature (°C)	16 µl H_2SO_4 (16%)	fungi	precipitation
47	4	10	RT	-	+	+
48	4	10	60	-	+	+
49	4	10	90	-	+	+
50	4	10	60	+	+	-
51	2	10	RT	-	-	+
52	2	10	60	-	+	+
53	2	10	90	-	+	+
54	2	10	60	+	-	+

Precipitation is already observed after 1 day. Fungi growth starts between day 1 and day 2. It seems the higher the concentration of copper, the faster the fungi grow and the more aggregates are formed. This could be a result of the fact that the concentration of reducing agents present in the extract is not high enough to reduce and stabilise this amount of copper at the same time.

8.3.4.2.2 Statistical analysis

A statistical analysis was performed to see which parameters (ratio copper/extract, temperature, addition of acid, storage in fridge, agitation) could have an effect on the presence of fungi and/or precipitation. SPSS software program for windows (release 23) was used fort he analysis with a significance level of 0,05 (confidence interval 95%) for all the tests. The statistical tests used were different from the ones used to analyse the compounds of the extracts because the results fort he extracts were quantitative (concentration polyphenols and reducing sugars) and the visual observations for the nanoparticles are qualitative (positive or negative).

First a crosstab analysis of the observation (fungi or precipitation) and each of the variables was calculated to determine if there could be a connection between the variable and the observation.

Then, to test whether there is a correlation between the two variables a chi-square test can be performed. If more than a quarter of the cells have an expected frequency of 5 or less, you are not allowed to interpret the result of the test. The crosstabs and consequent Chi-Square tests of the variables that have a positive effect on the observation are shown in this text. The tests for the variables that had a negative effect on the observation can be found in Annex II.

The crosstab and subsequent chi-square test for the effect of storage in the fridge on the presence of fungi can be seen in table 32 and 33.

Table 32: crosstab of variable storage in fridge on presence of fungi

Crosstab

			fridge		
			-	+	Total
funghi	-	Count	7	11	18
		Expected Count	14,4	3,6	18,0
		% of Total	11,7%	18,3%	30,0%
	+	Count	41	1	42
		Expected Count	33,6	8,4	42,0
		% of Total	68,3%	1,7%	70,0%
Total		Count	48	12	60
		Expected Count	48,0	12,0	60,0
		% of Total	80,0%	20,0%	100,0%

The crosstab shows the expected number of samples with or without presence of fungi versus the actual number of samples with or without the presence of fungi.

The chi-square test examines if the difference between the number of expected values and the actual values is significant. If the significance value of the Pearson Chi-Square is less then or equal to 0,05, the variables are related.

Table 33: Chi-Square test of variable storage in fridge on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	27,163[a]	1	,000		
Continuity Correction[b]	23,616	1	,000		
Likelihood Ratio	26,540	1	,000		
Fisher's Exact Test				,000	,000
N of Valid Cases	60				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 3,60.

b. Computed only for a 2x2 table

The Pearson value is in this case 0,000 so the storage of the samples in the fridge seems to have an effect of the presence of fungi.

The crosstab and subsequent Chi-Square test of variable agitation on the presence of fungi can be seen in table 34 and 35.

Table 34: crosstab of variable agitation on presence of fungi

Crosstab

			agitation		
			-	+	Total
funghi	-	Count	18	0	18
		Expected Count	10,8	7,2	18,0
		% of Total	30,0%	0,0%	30,0%
	+	Count	18	24	42
		Expected Count	25,2	16,8	42,0
		% of Total	30,0%	40,0%	70,0%
Total		Count	36	24	60
		Expected Count	36,0	24,0	60,0
		% of Total	60,0%	40,0%	100,0%

Table 35: Chi-Square test of variable agitation on the presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	17,143[a]	1	,000		
Continuity Correction[b]	14,845	1	,000		
Likelihood Ratio	23,397	1	,000		
Fisher's Exact Test				,000	,000
N of Valid Cases	60				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 7,20.

b. Computed only for a 2x2 table

The Pearson Chi-Square significance value is 0,000. The variable of agitation seems to have an influence on the presence of fungi.

The second correlation that is examined is the effect of the variables on the presence of precipitation.

The crosstab and subsequent Chi-Square test of variable temperature on the presence of precipitation can be seen in table 36 and 37.

Table 36: crosstab of variable temperature on the presence of precipitation

Crosstab

			Temperature			
			1	2	3	Total
precipitation	-	Count	1	11	9	21
		Expected Count	7,0	7,0	7,0	21,0
		% of Total	1,7%	18,3%	15,0%	35,0%
	+	Count	19	9	11	39
		Expected Count	13,0	13,0	13,0	39,0
		% of Total	31,7%	15,0%	18,3%	65,0%
Total		Count	20	20	20	60
		Expected Count	20,0	20,0	20,0	60,0
		% of Total	33,3%	33,3%	33,3%	100,0%

Table 37: Chi-Square test of variable temeprature on the presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	12,308[a]	2	,002
Likelihood Ratio	14,702	2	,001
N of Valid Cases	60		

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 7,00.

The Pearson Chi-Square significance value is 0,002. The variable of temperature seems to have an influence on the presence of precipitation.

The crosstab and subsequent Chi-Square test of variable agitation on the presence of precipitation can be seen in table 38 and 39.

Table 38: crosstab of variable agitation on the presence of precipitation

Crosstab

			agitation		
			-	+	Total
precipitation	-	Count	21	0	21
		Expected Count	12,6	8,4	21,0
		% of Total	35,0%	0,0%	35,0%
	+	Count	15	24	39
		Expected Count	23,4	15,6	39,0
		% of Total	25,0%	40,0%	65,0%
Total		Count	36	24	60
		Expected Count	36,0	24,0	60,0
		% of Total	60,0%	40,0%	100,0%

Table 39: Chi-Square test of variable agitation on the presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	21,538[a]	1	,000		
Continuity Correction[b]	19,051	1	,000		
Likelihood Ratio	28,792	1	,000		
Fisher's Exact Test				,000	,000
N of Valid Cases	60				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 8,40.

b. Computed only for a 2x2 table

The Pearson Chi-Square significance value is 0,000. The variable of temperature seems to have an influence on the presence of precipitation.

In general, the variables that seem to have an influence on the presence of fungi according to the statistical analysis are the storage of the samples in the fridge and agitation. This was expected because the temperature of the fridge slows down the metabolism of the micro-organisms and therefore the growth of the fungi. Agitation elevates the kinetic energy which promotes the growth of fungi. The surprising result is that the temperature and addition of acid don't seem to have any influence. The temperature usually increases the kinetic energy which should result in the growth of fungi and acid should slow down the growth.

The variables that seem to have an influence on the presence of precipitation according to the statistical analysis are temperature and agitation. Again this was expected. Both the temperature and agitation increase the contact between the particles which results in the formation of aggregates that precipitate because of their size. The results for storage in the fridge and addition of acid are not conclusive.

The variables that do not seem to have an influence on the presence of fungi are ratio copper/extract volume, addition of acid and temperature. This was not expected because copper supposedly has an anti-bacterial capacity but it is possible that the copper concentration used, is to low for this effect. The addition of acid should slow down the growth of fungi and the rise of temperature usually promotes fungi growth.

The variables that don't seem to have an effect on the presence of precipitation are ratio copper/extract volume, addition of acid and storage in the fridge, although the significance level for addition of acid and storage in the fridge are really close to 0,05 (see annex II). The addition of acid was not expected. A low pH usually prevents the precipitation of metals if the pH is below 5,5.

8.3.4.2.3 UV-Vis absorption spectra

The UV-Vis absorption spectra were measured after 1 hour, 2 days and 4 days. A 0,001 M solution of $CuSO_4$ was used as the source of copper. Selected spectra were used fort he representation of the effects of the variables.

The UV-Vis spectra of samples with variables ratio copper/extract, temperature, addition of acid and contact time can be seen in figure 47.

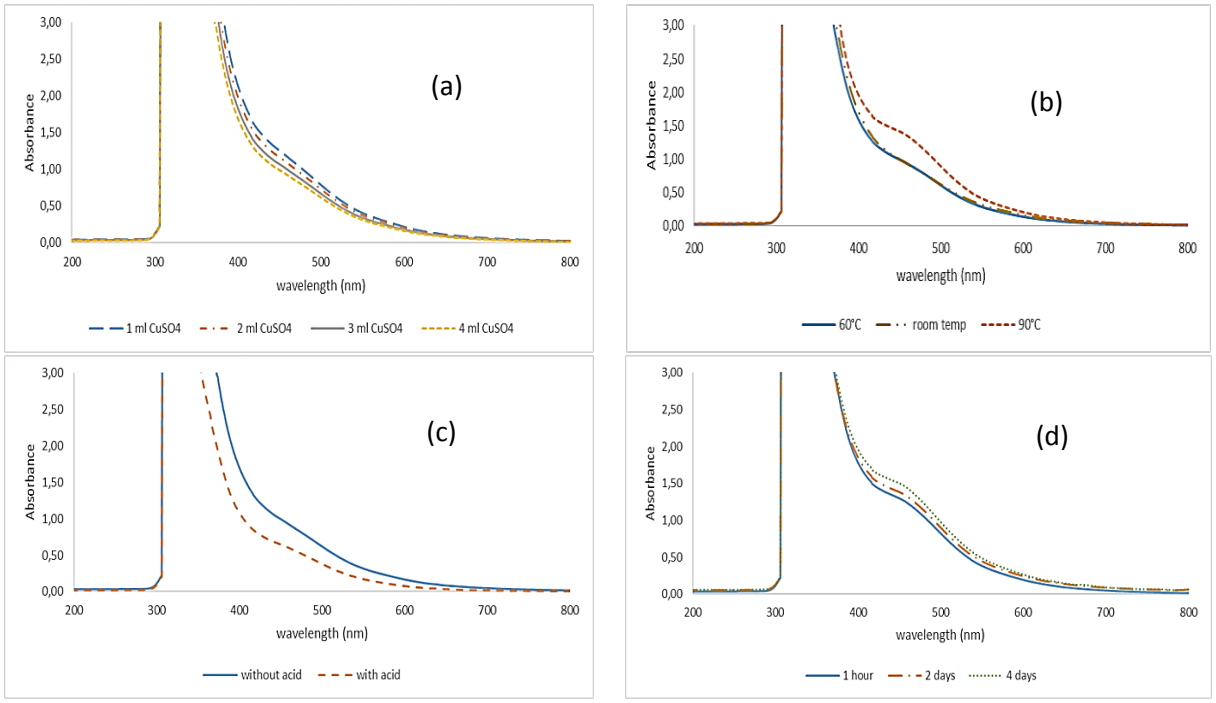


Figure 47: UV-Vis absorption spectra of variables ratio copper/extract volume (a), temperature (b), addition of acid (c) and contact time (d)

As can be seen in figure 47 (a), the ratio of copper/extract seems to have an influence on the absorption. The temperature (RT), addition of acid (no acid) and contact time (1 hour) were kept constant. It seems the higher the ratio, the lower the absorbance. The peak between 400-500 nm could be related to the formation of copper(I)oxide nanoparticles. The higher the amount of copper solution added, the higher the total volume. The same amount of copper nanoparticles in higher volume results in lower concentration which could explain the drop in absorbance.

Ratio copper/extract volume (4:10), contact time (1 hour), addition of acid (no acid) and volume of extract (10 ml) are constant for the spectra at different temperatures . The temperature has an effect on the absorbance (see figure 47 (b)). There is no difference between room temperature and 60°C but at 90°C the absorbance is much higher. This is to be expected because with higher temperatures, the kinetic energy rises which results in better contact between particles and solution.

The UV-Vis spectra for the effect of addition of acid of samples with 4 ml CuSO4 with and without acid (1 drop H2SO4 16%) can be seen in figure 47 (c). Ratio copper/extract volume (4:10), temperature (RT), contact time (1 hour) and extract volume (10 ml) are constant. The addition of a drop of acid (H2SO4 16%) also has an influence on the spectrum. It seems to slow down the reduction of copper and thereby the formation of nanoparticles.

Ratio copper/extract volume (6:10), temperature (90°C), addition of acid (no acid) and extract volume (10 ml) are constant for the spectra at different contact times. The longer the contact between copper(II)sulfate and the extract, the higher the absorbance (see figure 47 (d)). It seems that the reduction can take a long time. The organic compounds in the extract seem to need time to reduce and stabilise the nanoparticles formed.

Next are the UV-Vis spectra of samples with and without agitation with constant ratio copper/extract volume (3:10), temperature (RT), addition of acid (no acid), contact time (1 hour) and extract volume (10 ml) (see figure 48) .

The spectra of samples with $CuCl_2$ can be seen in figure 49. Ratio copper/extract volume (2:10), temperature (90°C), addition of acid (no acid), contact time (1 hour) and extract volume (10 ml) are constant.

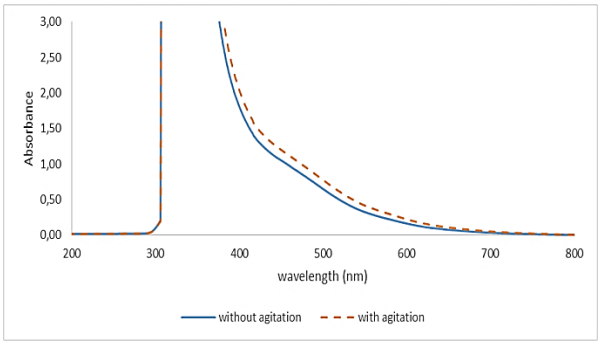


Figure 48: effect of agitation on sample with 3 ml CuSO4 in 10 ml of grape stalk extract at RT without acid for 1 hour

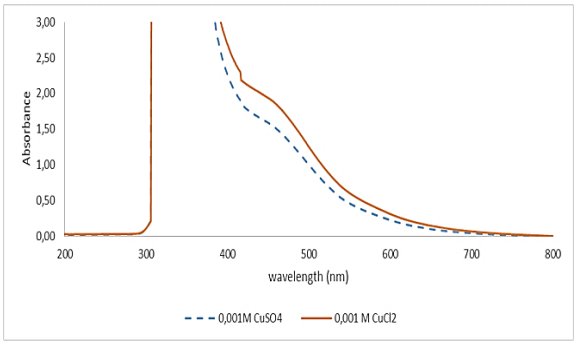


Figure 49: effect of use of 1 mM CuCl2 on sample with 2 ml CuCl2 in 10 ml of grape stalk extract at 90°C without acid after 1 hour

The absorbance seems higher with agitation (see figure 48). This is to be expected because agitation increases the contact between the particles and the solution. Which in turn increases the amount of particles formed. The problem with agitation is that it also promotes the formation of aggregates which results in precipitation.

The use of copper(II)chloride instead of copper(II)sulfate results in a higher absorbance which could mean an higher concentration of copper nanoparticles (see figure 49).

The UV-Vis spectra of samples with a higher concentration of copper can be seen in figure 50. Ratio copper/extract volume (4:10), temperature (60°C), addition of acid (no acid), contact time (1 hour) and extract volume (10 ml) are constant.

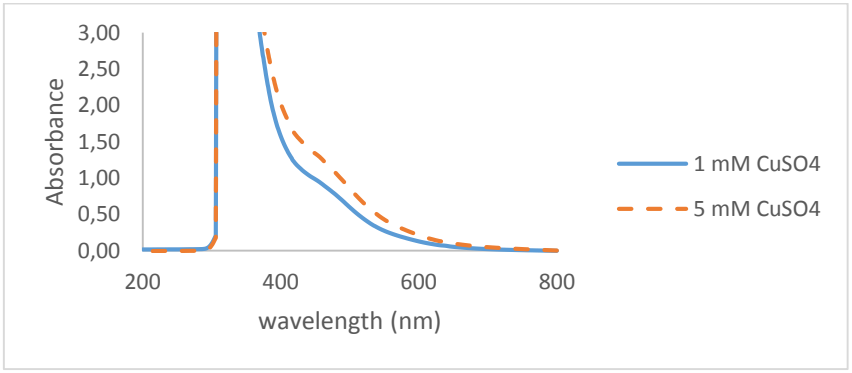


Figure 50: effect of 5 mM CuSO4 on sample with 4 ml CuSO4 in 10 ml of grape stalk extract at 60°C without acid after 1 hour

As can be seen in figure 50, the higher concentration of copper(II)sulfate solution results in a higher absorbance which could mean more copper nanoparticles. However, it also results in faster precipitation as was observed after 4 days (see table 31).

8.3.4.2.4 EDX/SEM

SEM analysis was performed on samples with ratio copper/extract of 1:10, temperature at RT (sample 11), 60°C (sample 19) and 90°C (sample 23) without agitation. The sample at 60°C was analysed twice. The first sample was without agitation and the second with agitation. The samples were first measured without centrifugation.

The SEM results of the samples without centrifugation showed that the images were not very clear. The pores of the polycarbonate filter were not only covered with too much particles but it seemed that the extract also interfered with the image. The decision was made to centrifuge the samples and wash and resuspend the pellet once with milli-Q water before analysis.

The SEM images of sample 11 without centrifugation at 1000x magnification can be seen in figure 51 and with centrifugation in figure 52.

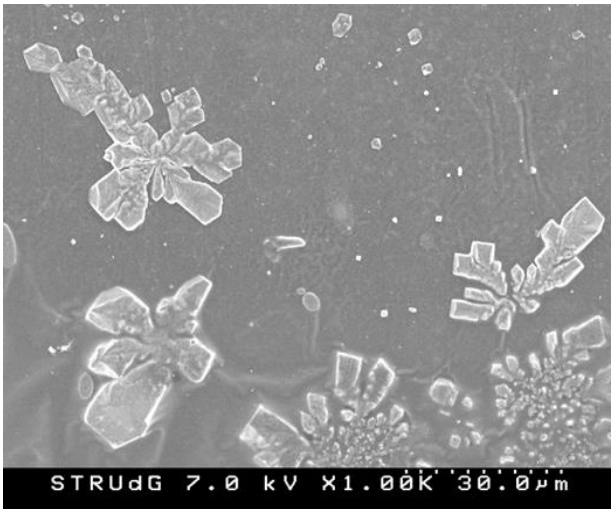


Figure 51: SEM image of sample 1:10 at RT not centrifuged at 1000x magnification

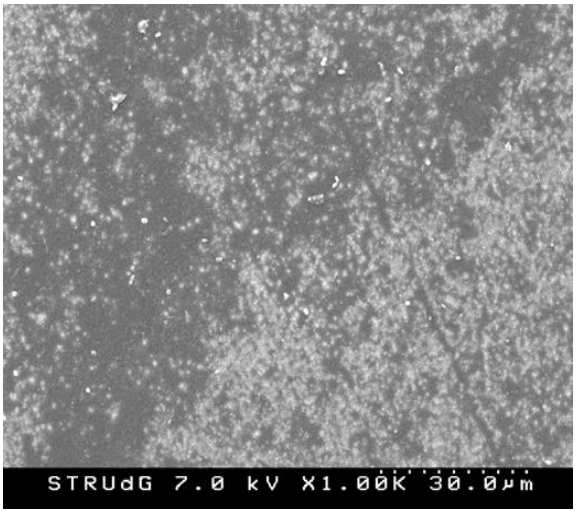


Figure 52: SEM image of sample 1:10 at RT centrifuged at 1000x magnification

As can be seen in figure 51, crystals are present in the sample. These crystals are almost gone after centrifugation.
The results of the SEM analysis of samples 11, 19 (with and without agitation) and 23 can be seen in figures 53-56.

As can be seen in figures 53-56, the image is much more clear. The pores of the polycarbonate filter are more visible. In figure 53, the formation of aggregates is apparent because of the rough edges and the size of the particles. At 60°C (figure 54), aggregates are also visible but a lot less then with the sample at room temperature. Isolated particles can also be seen. The general shape of the particles seems spherical. At 90°C (figure 55) more isolated particles can be seen. Almost no aggregates are present. The sample with agitation (figure 56) clearly shows the most aggregates formed. They are also the biggest particles present. This can be explained by the fact that agitation increases the contact which results in faster formation of bigger particles.

Additional SEM images can be found in Annex I.

Energy dispersive x-ray spectroscopy was performed to determine the elemental composition of the nanoparticles. More specificly to see if copper is present. This time it was focused on 1 specific point of the sample were it was believed nanoparticles were present.

EDX analysis was performed on sample with ratio copper/extract volume 1:10 at 60°C for 1 hour. First without centrifugation by putting 1 ml of sample on the filter and drying overnight in the oven at 50°C. The next sample was centrifuged at 20 000 rpm for 10 minutes. The pellet was washed with Milli-Q and redispersed. 1 ml of the redispersed pellet was put on the filter and dried in the oven overnight.

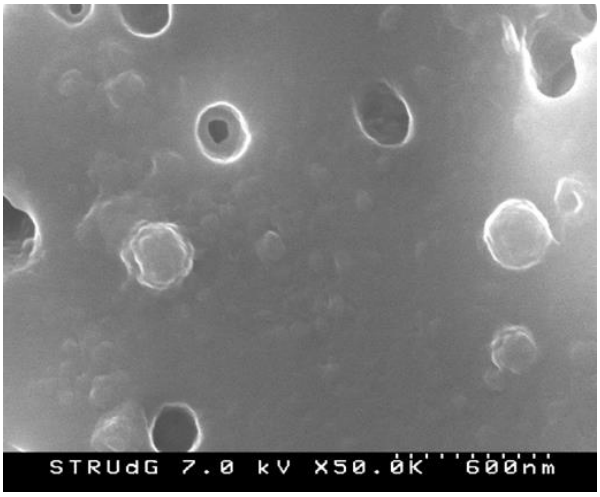


Figure 53: SEM image of sample 1:10 at RT, centrifuged and at 50 000x magnification

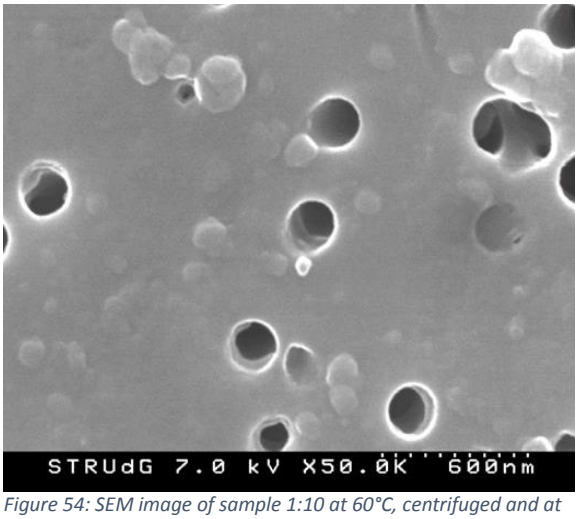


Figure 54: SEM image of sample 1:10 at 60°C, centrifuged and at 50 000x magnification

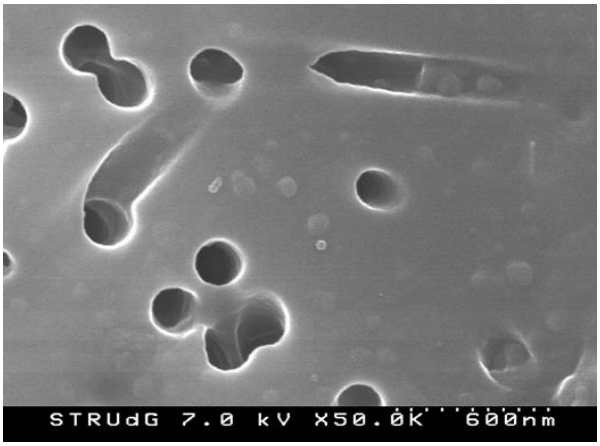


Figure 55: SEM image of sample 1:10 at 90°C, centrifuged and at 50 000x magnification

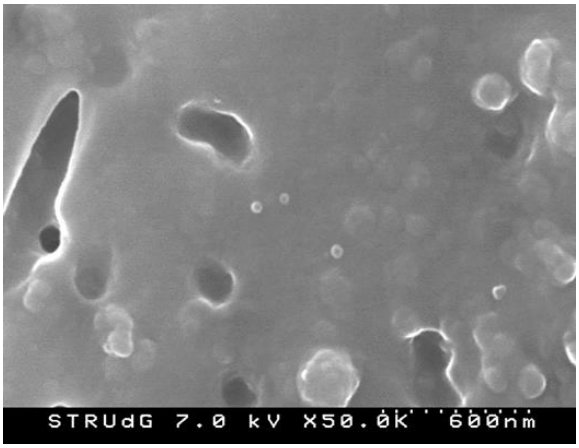


Figure 56: SEM image of sample 1:10 at 60°C with agitation, centrifuged and at 50 000x magnification

The results of the EDX analysis of the sample without centrifugation can be seen in figure 57. The results of the sample after centrifugation can be seen in figure 58.

For both samples, the EDX reports show no presence of copper (element)(see figure 57-58). This means that either that there are no copper nanoparticles present or that the concentration of nanoparticles present is too low to detect. The concentration of carbon in the samples is really high. This is because the extract contains a lot of organic compounds which could also be seen in the UV-Vis absorption spectra (peak between 300-400 nm) (see figure 47-49).

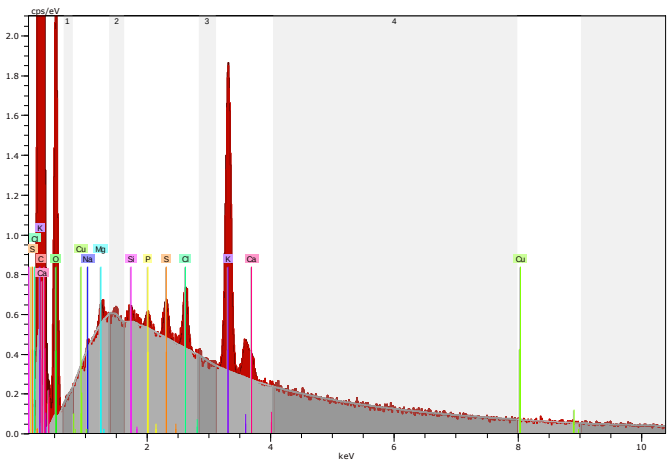
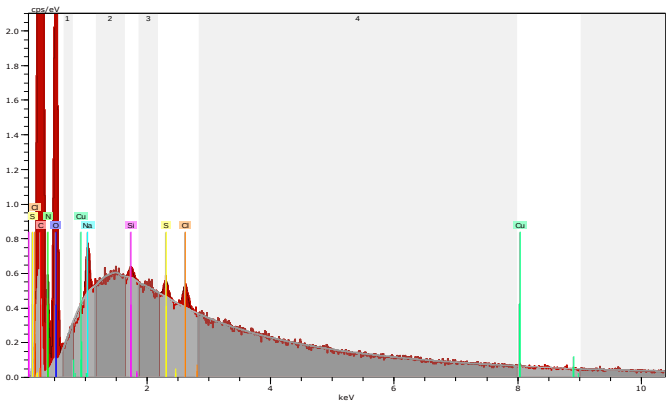


Figure 57: EDX report of sample 1:10 at 60°C for 1 hour without centrifugation

Spectrum: 4 40 60° 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]
Carbono	K-series	86,26	86,26	90,01
Oxígeno	K-series	11,99	11,99	9,39
Sodio	K-series	0,02	0,02	0,01
Magnesio	K-series	0,06	0,06	0,03
Silicio	K-series	0,03	0,03	0,01
Fósforo	K-series	0,04	0,04	0,02
Azufre	K-series	0,09	0,09	0,04
Cloro	K-series	0,17	0,17	0,06
Potasio	K-series	1,23	1,23	0,39
Calcio	K-series	0,11	0,11	0,03
Cobre	K-series	0,00	0,00	0,00
Total:		100,00	100,00	100,00



Spectrum: 4 40 60° centrif 1

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]
Carbono	K-series	80,87	80,87	84,47
Nitrógeno	K-series	5,60	5,60	5,02
Oxígeno	K-series	13,23	13,23	10,38
Sodio	K-series	0,15	0,15	0,08
Silicio	K-series	0,02	0,02	0,01
Azufre	K-series	0,04	0,04	0,01
Cloro	K-series	0,06	0,06	0,02
Cobre	K-series	0,02	0,02	0,00
Total:		100,00	100,00	100,00

Figure 58: EDX report of sample 1:10 at 60°C for 1 hour after centrifugation

8.4 Summary

In order to obtain copper metal nanoparticles, several variables have to be controlled. The results of the UV-Vis absorption spectra show that an increase in temperature, longer contact time, agitation and a higher copper concentration have a positive influence on the formation of copper nanoparticles. But the results of the SEM analysis and the statistical analysis show that some of these variables (temperature and agitation) cause faster precipitation.

9 General conclusion

Preliminary results to obtain spent coffee and grape stalk extracts showed that (I) the highest concentration of polyphenolic compounds was obtained for 3 grams of waste with particle size of 100-250 µm in 50 ml Milli-Q at 100°C ; (II) the concentration of polyphenols was 10 times higher for grape stalk extract than spent coffee extract.

The study of the influence of temperature and contact time on the concentration of polyphenolic compounds and reducing sugars in grape stalk extracts showed that the temperature had an influence on the polyphenols but not on the reducing sugars and that contact time had an influence on both the polyphenols and reducing sugars. These results were confirmed by a statistical analysis.

The total organic carbon (TOC) analysis did not provide any conclusive results concerning the effect of the studied variables on the total organic carbon content.

Preliminary experiments performed to obtain copper nanoparticles using grape stalk extracts allowed to put into evidence: (I) UV/Vis analysis showed a peak between 400-600 nm, (II) SEM analysis demonstrated aggregates of nanoparticles and (III) EDX analysis detected the presence of copper.

The study of the influence of several variables on copper nanoparticles synthesis using spent coffee provided the following results: (I) visual observations indicated the presence of funghi in only one of the samples and that in general temperature and addition of acid promoted the formation of precipitation ; (II) A statistical analysis of the visual observations confirmed the effect of addition of acid but not the effect of temperature and (III) UV/Vis spectra didn't show the characteristic peak between 400-600 nm.

The study of the influence of several variables on copper nanoparticles synthesis using grape stalk provided the following results: (I) temperature, agitation, addition of acid, copper(II)chloride and copper(II)sulfate (0,005 M) increased the amount of fungi while storage in the fridge decreased it; (II) agitation and copper(II)sulfate (0,005 M) promoted the precipitation while storage in the fridge, temperature, addition of acid and copper(II)chloride decreased the chance of precipitation ; (III) statistical analysis confirmed the effect of storage in the fridge, and agitation on fungi growth, statistics also confirmed the effect of temperature and agitation on precipitation; (IV) UV/Vis spectra showed the presence of a peak between 400-600 nm for all the variables studied except for adition of acid; (V) SEM analysis presented more clear images after centrifugation and washing of samples ; (VI) EDX analysis did not detect the presence of copper ; (VII) the color of the solution was observed to change to red when the synthesis was performed at high temperatures indicating a possible formation of copper(I)oxide.

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ANNEX I—SEM IMAGES

Preliminary samples of copper nanoparticles using grape stalk extract

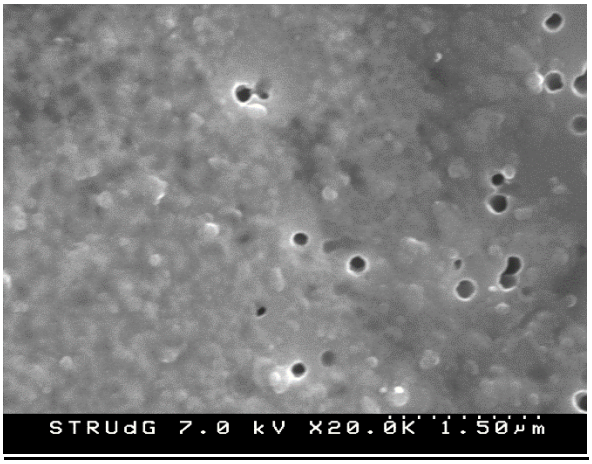

Figure 59: SEM image of sample 1:100 at 20 000x magnification

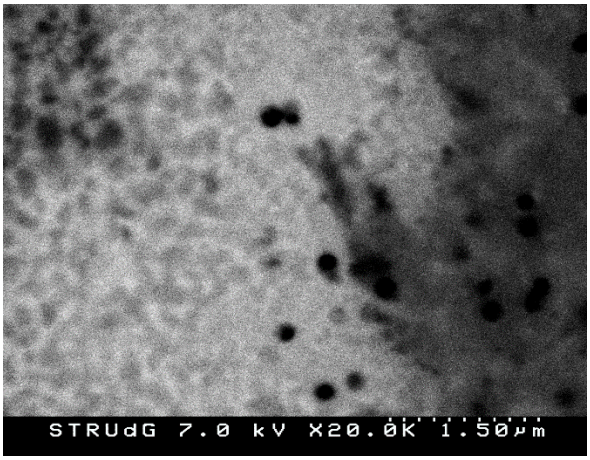

Figure 60: SEM image of sample 1:100, backscatter at 20 000x magnification

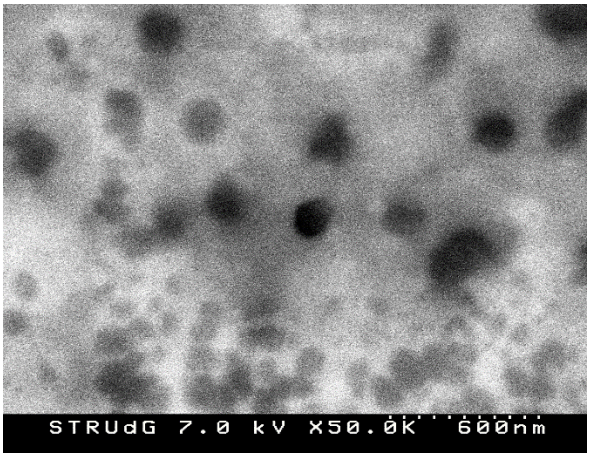

Figure 61: SEM image of sample 1:100, backscatter at 50 000x magnification

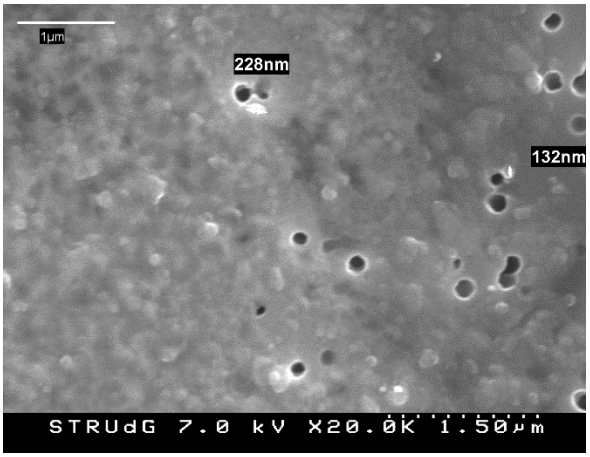

Figure 62: SEM image of sample, size analysis at 20 000x magnification

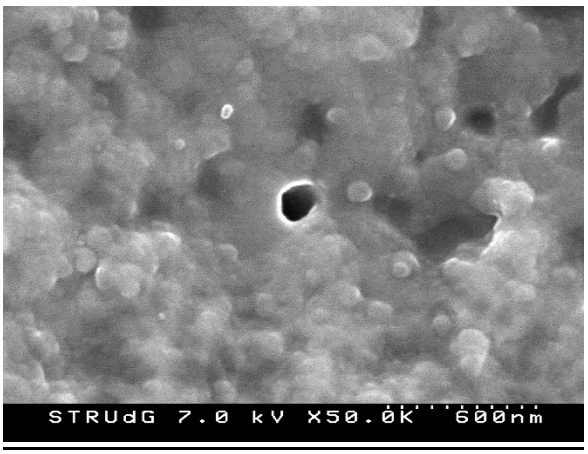

Figure 63: SEM image of sample 1:100, secondary electrons at 50 000x magnification

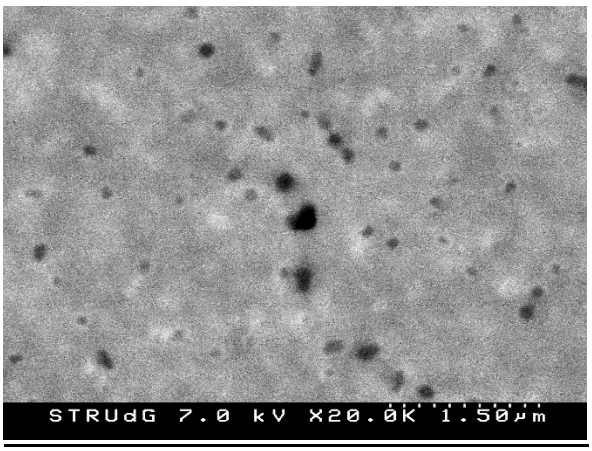

Figure 64: SEM image of sample 30:170 (1), backscatter at 20 000x magnification

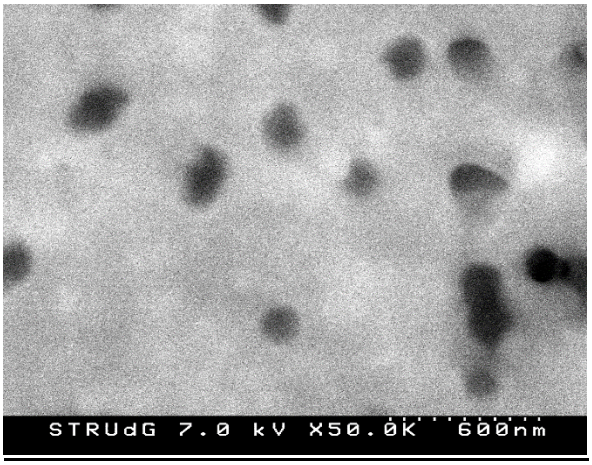

Figure 65: SEM image of sample 30:170 (1), backscatter at 50 000x magnification

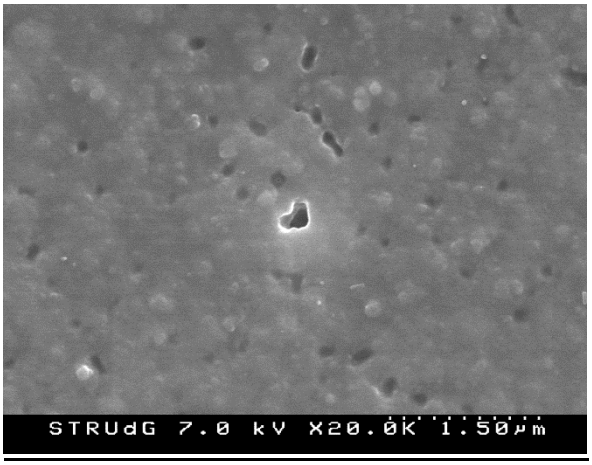

Figure 66: SEM image of sample 30:170 (1), secondary electrons at 20 000x magnification

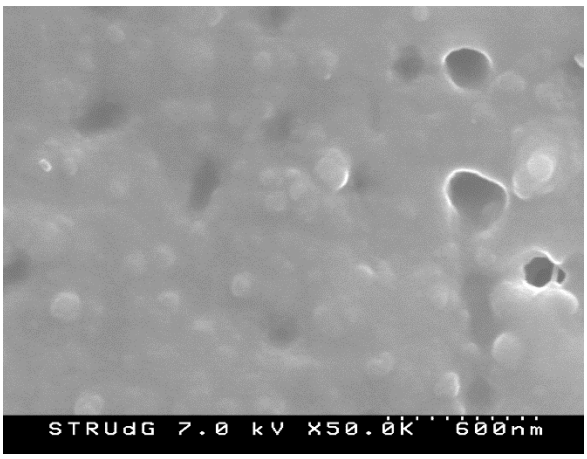

Figure 67: SEM image of sample 30:170 (1), secondary electrons at 50 000x magnification

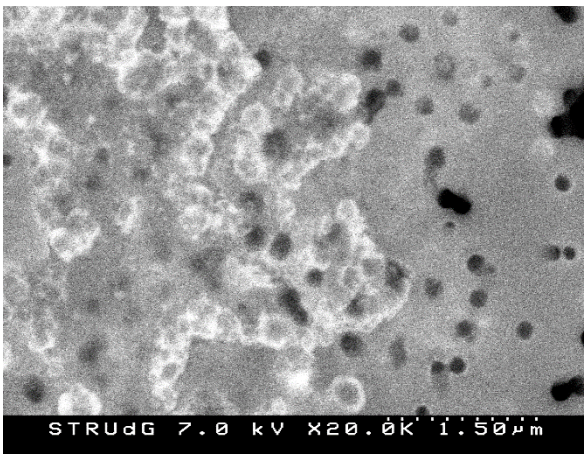

Figure 68: SEM image of sample 30:170 (2), backscatter at 20 000x magnification

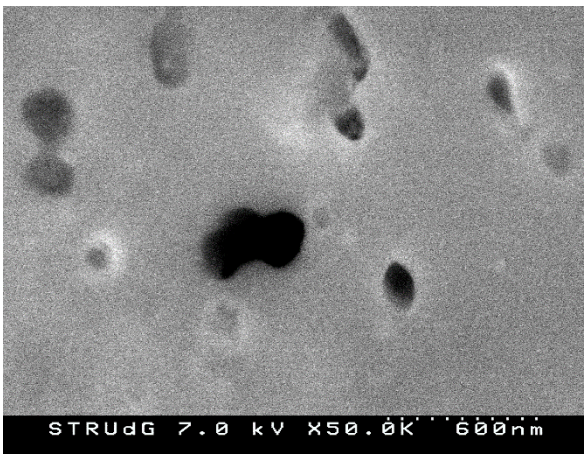

Figure 69: SEM image of sample 30:170 (2), backscatter at 50 000x magnification

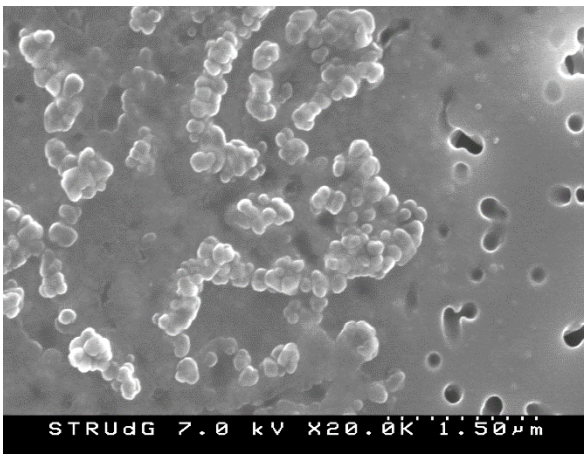

Figure 70: SEM image of sample 30:170 (2), secondary electrons at 20 000x magnification

Samples 1:10 of copper nanoparticles using grape stalk extract

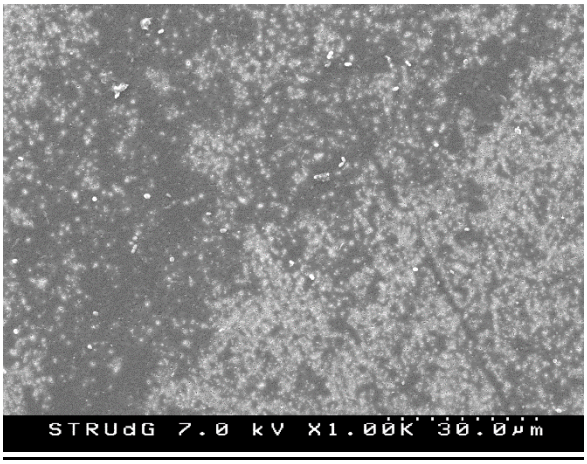

Figure 71: SEM image of sample 1:10 at RT, centrifuged, at 1000x magnification

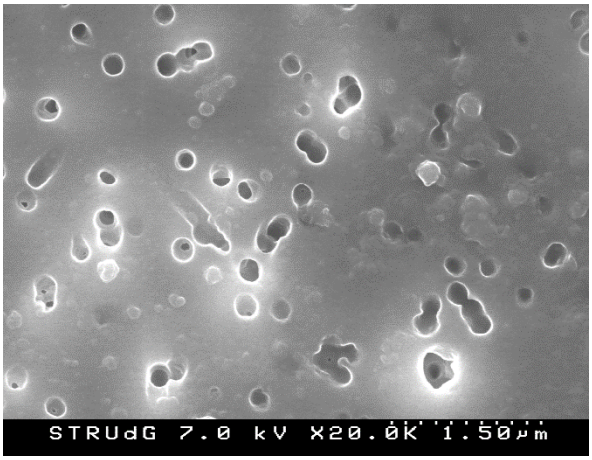

Figure 72: SEM image of sample 1:10 at RT, centrifuged, at 20 000x magnification

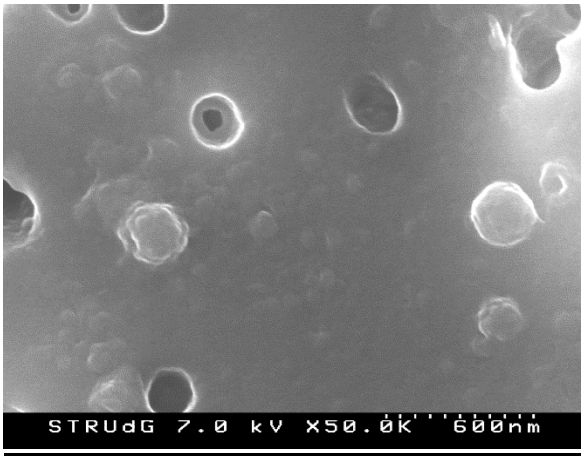

Figure 73: SEM image of sample 1:10 at RT, centrifuged, at 50 000x magnification

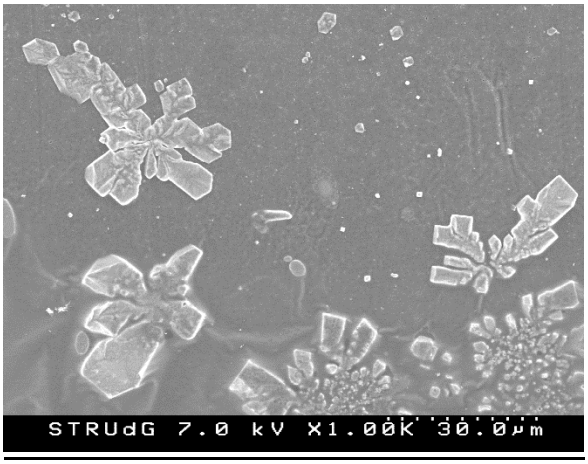

Figure 74: SEM image of sample 1:10 at RT, not centrifuged, at 1000x magnification

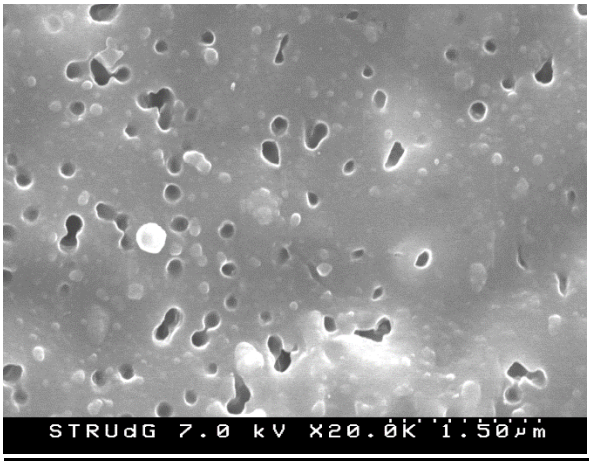

Figure 75: SEM image of sample 1:10 at RT, not centrifuged, at 20 000x magnification

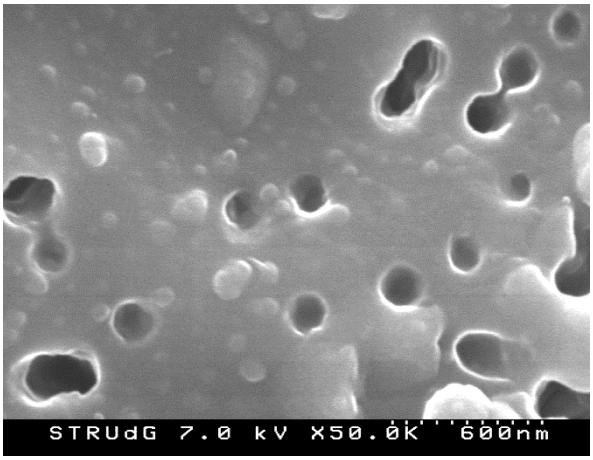

Figure 76: SEM image of sample 1:10 at RT, not centrifuged, at 50 000x magnification

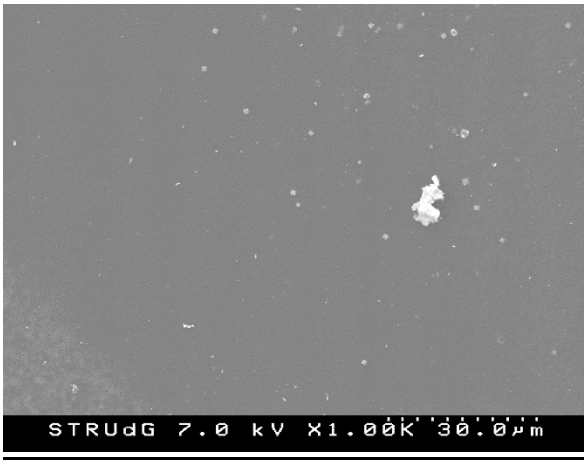

Figure 77: SEM image of sample 1:10 at 60°C with agitation, not centrifuged, at 1000x magnification

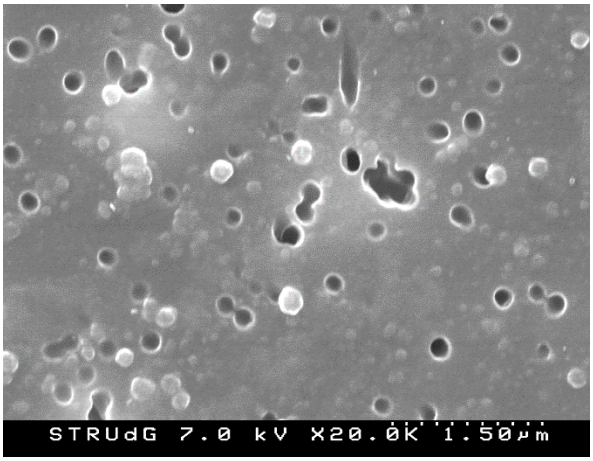

Figure 78: SEM image of sample 1:10 at 60°C with agitation, not centrifuged, at 20 000x magnification

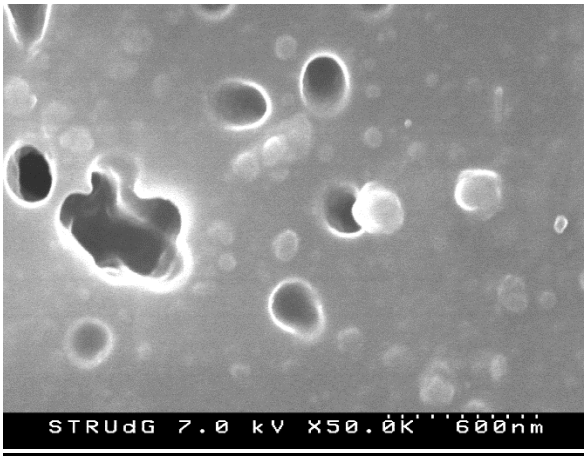

Figure 79: SEM image of sample 1:10 at 60°C with agitation, not centrifuged, at 50 000x magnification

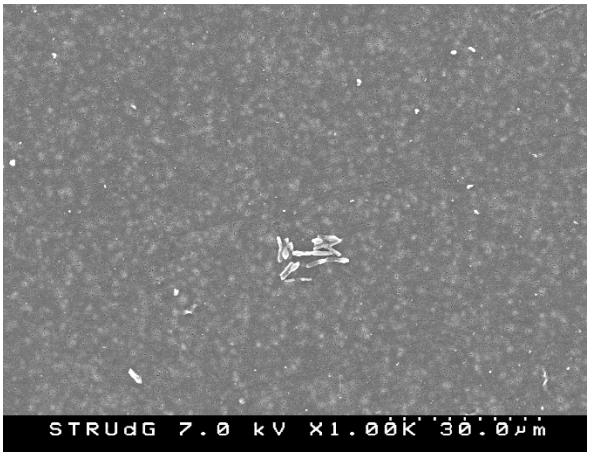

Figure 80: SEM image of sample 1:10 at 60°C, not centrifuged, at 1000x magnification

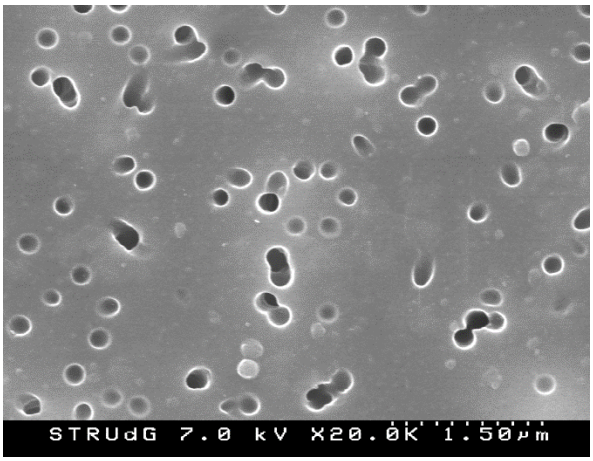

Figure 81: SEM image of sample 1:10 at 60°C, not centrifuged, at 20 000x magnification

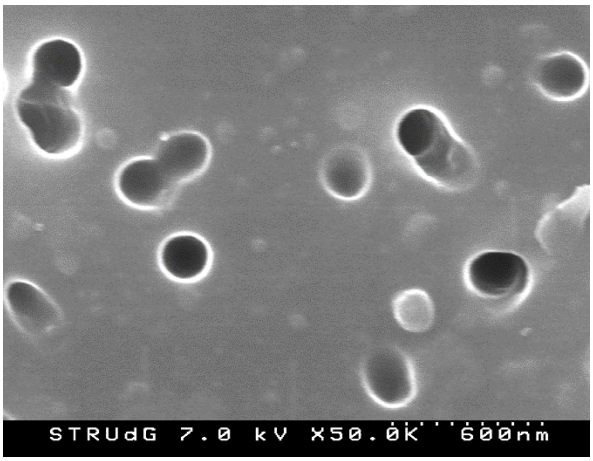

Figure 82: SEM image of sample 1:10 at 60°C, not centrifuged, at 50 000x magnification

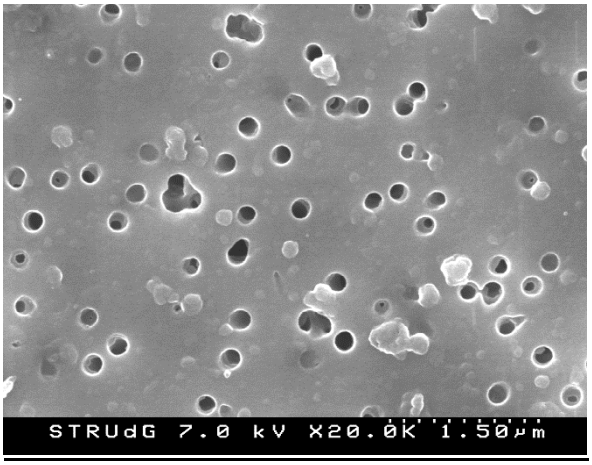

Figure 83: SEM image of sample 1:10 at 60°C, centrifuged, at 20 000x magnification

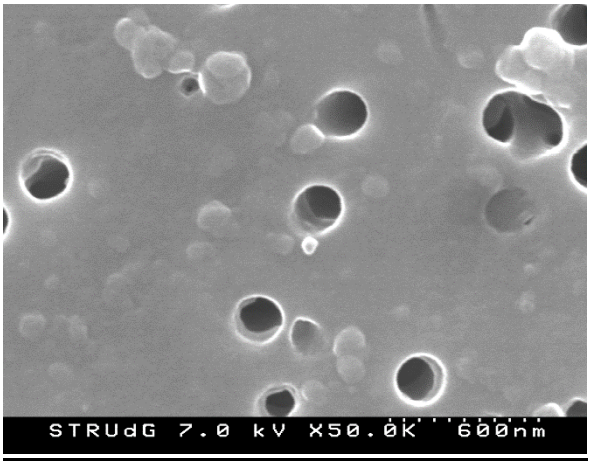

Figure 84: SEM image of sample 1:10 at 60°C, centrifuged, at 50 000x magnification

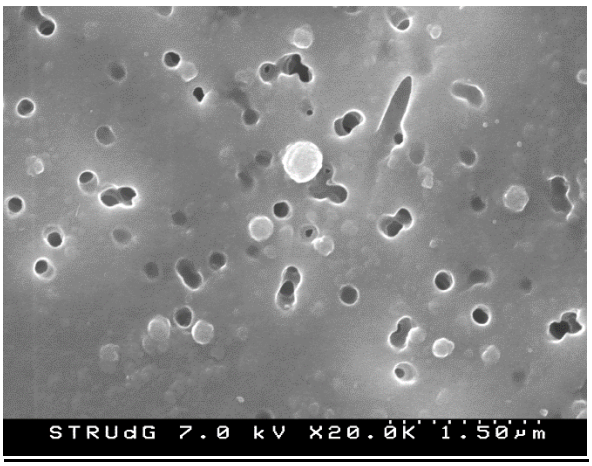

Figure 85: SEM image of sample 1:10 at 60°C with agitation, centrifuged, at 20 000x magnification

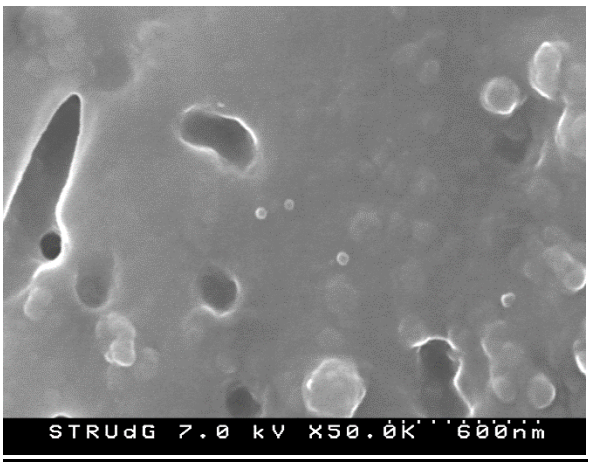

Figure 86: SEM image of sample 1:10 at 60°C with agitation, centrifuged, at 50 000x magnification

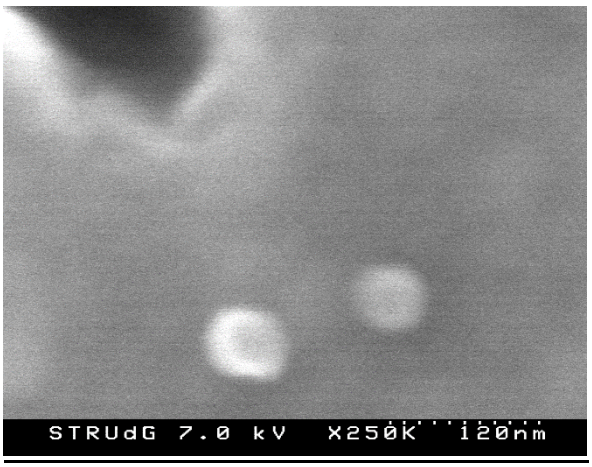

Figure 87: SEM image of sample 1:10 at 60°C with agitation, centrifuged, at 250 000x magnification

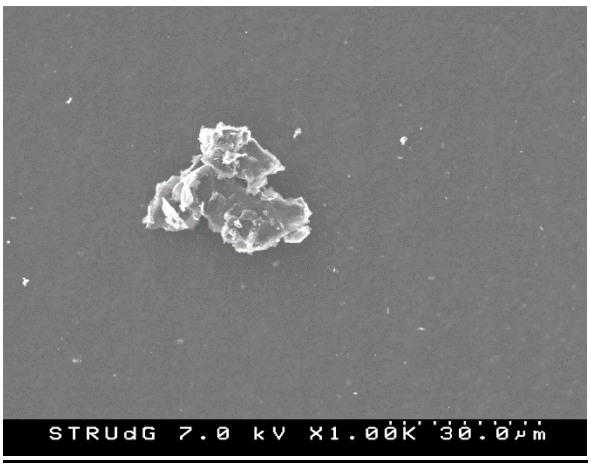

Figure 88: SEM image of sample 1:10 at 90°C, not centrifuged, at 1000x magnification

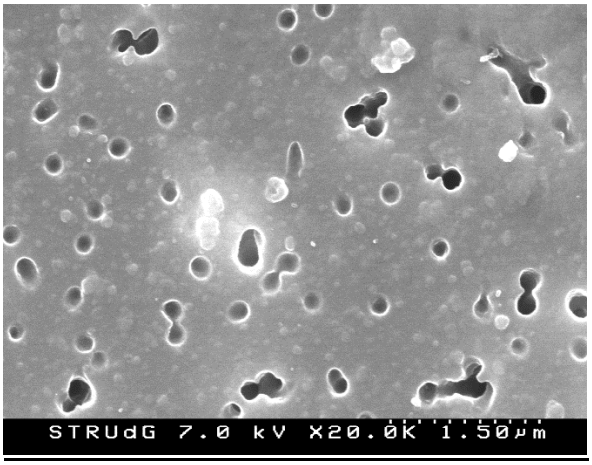

Figure 89: SEM image of sample 1:10 at 90°C, not centrifuged, at 20 000x magnification

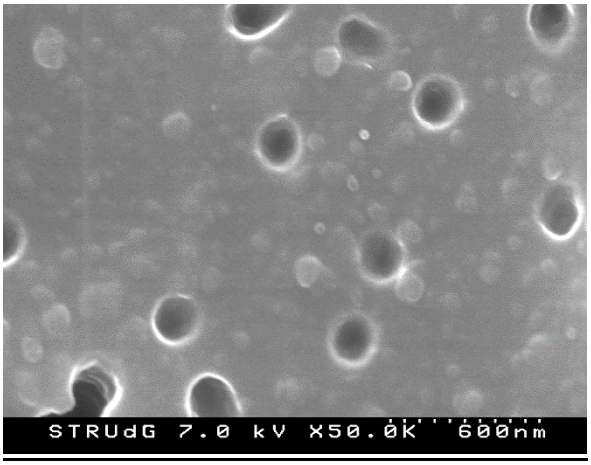

Figure 90: SEM image of sample 1:10 at 90°C, not centrifuged, at 50 000x magnification

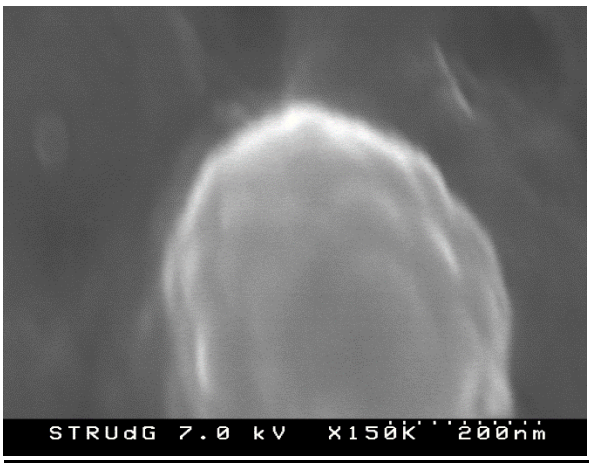

Figure 91: SEM image of sample 1:10 at 90°C, centrifuged, at 150 000x magnification

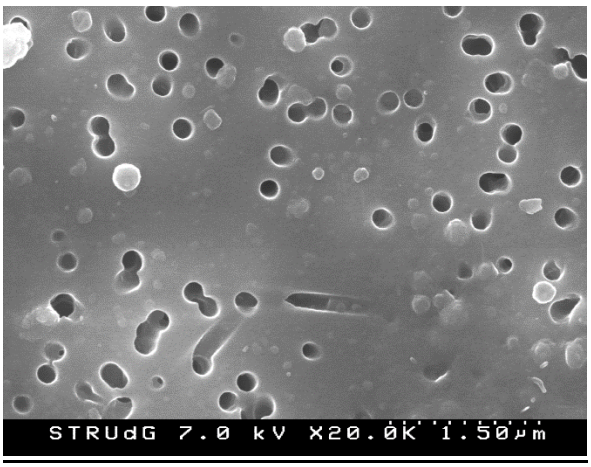

Figure 92: SEM image of sample 1:10 at 90°C, centrifuged, at 20 000x magnification

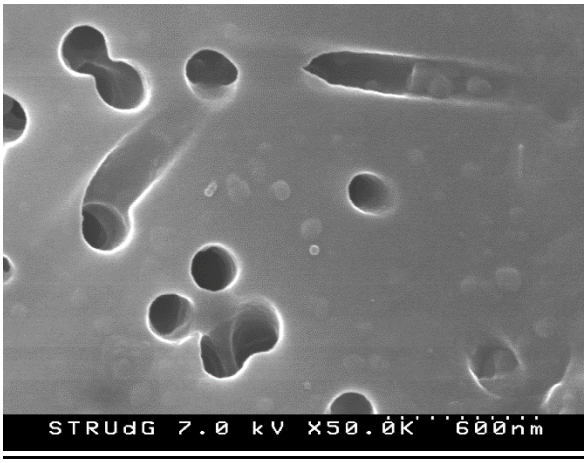

Figure 93: SEM image of sample 1:10 at 90°C, centrifuged, at 50 000x magnification

ANNEX II—tables of statistical analysis of visual observations

Crosstabs and Chi-Square tests of spent coffee

fungi * copper solution (ml)

Table 40: Crosstab for variable ratio copper/extract volume on presence of fungi

Crosstab

			copper solution (ml)				
			1,0	2,0	4,0	6,0	Total
fungi	-	Count	5	6	6	6	23
		Expected Count	5,8	5,8	5,8	5,8	23,0
		% of Total	20,8%	25,0%	25,0%	25,0%	95,8%
	+	Count	1	0	0	0	1
		Expected Count	,3	,3	,3	,3	1,0
		% of Total	4,2%	0,0%	0,0%	0,0%	4,2%
Total		Count	6	6	6	6	24
		Expected Count	6,0	6,0	6,0	6,0	24,0
		% of Total	25,0%	25,0%	25,0%	25,0%	100,0%

Table 41: Chi-square test of variable ratio copper/extract volume on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	3,130[a]	3	,372
Likelihood Ratio	2,907	3	,406
N of Valid Cases	24		

a. 4 cells (50,0%) have expected count less than 5. The minimum expected count is ,25.

fungi * temperature (°C)

Table 42: Crosstab of variable temperature on presence of fungi

Crosstab

			temperature (°C)			
			60	90	RT	Total
fungi	-	Count	8	8	7	23
		Expected Count	7,7	7,7	7,7	23,0
		% of Total	33,3%	33,3%	29,2%	95,8%
	+	Count	0	0	1	1
		Expected Count	,3	,3	,3	1,0
		% of Total	0,0%	0,0%	4,2%	4,2%
Total		Count	8	8	8	24
		Expected Count	8,0	8,0	8,0	24,0
		% of Total	33,3%	33,3%	33,3%	100,0%

Table 43: Chi-square test of variable temperature on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	2,087[a]	2	,352
Likelihood Ratio	2,286	2	,319
N of Valid Cases	24		

a. 3 cells (50,0%) have expected count less than 5. The minimum expected count is ,33.

fungi * H2SO4 (16%)

Table 44: Crosstab of variable addition of acid on presence of fungi

Crosstab

			H2SO4 (16%)		
			-	+	Total
fungi	-	Count	11	12	23
		Expected Count	11,5	11,5	23,0
		% of Total	45,8%	50,0%	95,8%
	+	Count	1	0	1
		Expected Count	,5	,5	1,0
		% of Total	4,2%	0,0%	4,2%
Total		Count	12	12	24
		Expected Count	12,0	12,0	24,0
		% of Total	50,0%	50,0%	100,0%

Table 45: Chi-square test of variable addition of acid on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1,043[a]	1	,307		
Continuity Correction[b]	,000	1	1,000		
Likelihood Ratio	1,430	1	,232		
Fisher's Exact Test				1,000	,500
N of Valid Cases	24				

a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is ,50.

b. Computed only for a 2x2 table

precipitation * copper solution (ml)

Table 46: crosstab of variable ratio copper/extract volume on presence of precipitation

Crosstab

			copper solution (ml)				
			1,0	2,0	4,0	6,0	Total
precipitation	-	Count	3	1	1	2	7
		Expected Count	1,8	1,8	1,8	1,8	7,0
		% of Total	12,5%	4,2%	4,2%	8,3%	29,2%
	+	Count	3	5	5	4	17
		Expected Count	4,3	4,3	4,3	4,3	17,0
		% of Total	12,5%	20,8%	20,8%	16,7%	70,8%
Total		Count	6	6	6	6	24
		Expected Count	6,0	6,0	6,0	6,0	24,0
		% of Total	25,0%	25,0%	25,0%	25,0%	100,0%

Table 47: Chi-square test of variable ratio copper/extract volume on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	2,218[a]	3	,528
Likelihood Ratio	2,205	3	,531
N of Valid Cases	24		

a. 8 cells (100,0%) have expected count less than 5. The minimum expected count is 1,75.

precipitation * temperature (°C)

Table 48: crosstab of variable temperature on presence of precipitation

Crosstab

			temperature (°C)			
			60	90	RT	Total
precipitation	-	Count	2	1	4	7
		Expected Count	2,3	2,3	2,3	7,0
		% of Total	8,3%	4,2%	16,7%	29,2%
	+	Count	6	7	4	17
		Expected Count	5,7	5,7	5,7	17,0
		% of Total	25,0%	29,2%	16,7%	70,8%
Total		Count	8	8	8	24
		Expected Count	8,0	8,0	8,0	24,0
		% of Total	33,3%	33,3%	33,3%	100,0%

Table 49: Chi-square test of variable temperature on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	2,824[a]	2	,244
Likelihood Ratio	2,859	2	,239
N of Valid Cases	24		

a. 3 cells (50,0%) have expected count less than 5. The minimum expected count is 2,33.

Crosstabs and Chi-Square tests of grape stalk

funghi * CuSO4 (ml)

Table 50: Crosstab of variable copper/extract volume on presence of fungi

Crosstab

			CuSO4 (ml)				
			1,0	2,0	4,0	6,0	Total
funghi	-	Count	1	5	7	5	18
		Expected Count	3,6	4,5	5,4	4,5	18,0
		% of Total	1,7%	8,3%	11,7%	8,3%	30,0%
	+	Count	11	10	11	10	42
		Expected Count	8,4	10,5	12,6	10,5	42,0
		% of Total	18,3%	16,7%	18,3%	16,7%	70,0%
Total		Count	12	15	18	15	60
		Expected Count	12,0	15,0	18,0	15,0	60,0
		% of Total	20,0%	25,0%	30,0%	25,0%	100,0%

Table 51: Chi-square test of variable copper/extract volume on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	3,519[a]	3	,318
Likelihood Ratio	4,172	3	,243
N of Valid Cases	60		

a. 3 cells (37,5%) have expected count less than 5. The minimum expected count is 3,60.

funghi * H2SO4

Table 52: Crosstab of variable addition of acid on presence of fungi

Crosstab

			H2SO4		
			-	+	Total
funghi	-	Count	12	6	18
		Expected Count	9,9	8,1	18,0
		% of Total	20,0%	10,0%	30,0%
	+	Count	21	21	42
		Expected Count	23,1	18,9	42,0
		% of Total	35,0%	35,0%	70,0%
Total		Count	33	27	60
		Expected Count	33,0	27,0	60,0
		% of Total	55,0%	45,0%	100,0%

Table 53: Chi-square test of variable addition of acid on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1,414[a]	1	,234		
Continuity Correction[b]	,821	1	,365		
Likelihood Ratio	1,438	1	,230		
Fisher's Exact Test				,270	,183
N of Valid Cases	60				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 8,10.

b. Computed only for a 2x2 table

funghi * Temperature

Table 54: Crosstab of variable temperature on presence of fungi

Crosstab

			Temperature			
			1	2	3	Total
funghi	-	Count	8	7	3	18
		Expected Count	6,0	6,0	6,0	18,0
		% of Total	13,3%	11,7%	5,0%	30,0%
	+	Count	12	13	17	42
		Expected Count	14,0	14,0	14,0	42,0
		% of Total	20,0%	21,7%	28,3%	70,0%
Total		Count	20	20	20	60
		Expected Count	20,0	20,0	20,0	60,0
		% of Total	33,3%	33,3%	33,3%	100,0%

Table 55: Chi-square test of variable temperature on presence of fungi

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	3,333[a]	2	,189
Likelihood Ratio	3,577	2	,167
N of Valid Cases	60		

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 6,00.

precipitation * CuSO4 (ml)

Table 56: Crosstab of variable copper/extract volume on presence of precipitation

Crosstab

			CuSO4 (ml)				
			1,0	2,0	4,0	6,0	Total
precipitation	-	Count	3	5	6	7	21
		Expected Count	4,2	5,3	6,3	5,3	21,0
		% of Total	5,0%	8,3%	10,0%	11,7%	35,0%
	+	Count	9	10	12	8	39
		Expected Count	7,8	9,8	11,7	9,8	39,0
		% of Total	15,0%	16,7%	20,0%	13,3%	65,0%
Total		Count	12	15	18	15	60
		Expected Count	12,0	15,0	18,0	15,0	60,0
		% of Total	20,0%	25,0%	30,0%	25,0%	100,0%

Table 57: Chi-square test of variable copper/extract volume on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	1,465[a]	3	,690
Likelihood Ratio	1,460	3	,692
N of Valid Cases	60		

a. 1 cells (12,5%) have expected count less than 5. The minimum expected count is 4,20.

precipitation * H2SO4

Table 58: Crosstab of variable addition of acid on presence of precipitation

Crosstab

			H2SO4		
			-	+	Total
precipitation	-	Count	15	6	21
		Expected Count	11,5	9,5	21,0
		% of Total	25,0%	10,0%	35,0%
	+	Count	18	21	39
		Expected Count	21,5	17,6	39,0
		% of Total	30,0%	35,0%	65,0%
Total		Count	33	27	60
		Expected Count	33,0	27,0	60,0
		% of Total	55,0%	45,0%	100,0%

Table 59: Chi-square test of variable addition of acid on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3,523[a]	1	,061		
Continuity Correction[b]	2,576	1	,108		
Likelihood Ratio	3,615	1	,057		
Fisher's Exact Test				,102	,053
N of Valid Cases	60				

a. 0 cells (0,0%) have expected count less than 5. The minimum expected count is 9,45.

b. Computed only for a 2x2 table

precipitation * fridge

Table 60: Crosstab of variable storage in the fridge on presence of precipitation

Crosstab

			fridge		
			-	+	Total
precipitation	-	Count	14	7	21
		Expected Count	16,8	4,2	21,0
		% of Total	23,3%	11,7%	35,0%
	+	Count	34	5	39
		Expected Count	31,2	7,8	39,0
		% of Total	56,7%	8,3%	65,0%
Total		Count	48	12	60
		Expected Count	48,0	12,0	60,0
		% of Total	80,0%	20,0%	100,0%

Table 61: Chi-square test of variable storage in the fridge on presence of precipitation

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3,590[a]	1	,058		
Continuity Correction[b]	2,422	1	,120		
Likelihood Ratio	3,444	1	,063		
Fisher's Exact Test				,090	,062
N of Valid Cases	60				

a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 4,20.

b. Computed only for a 2x2 table