"POLITEHNICA" UNIVERSITY OF BUCHAREST
Doctoral School of Applied Chemistry and Materials Science
Department of Chemical and Biochemical Engineering

DOCTORAL THESIS

IMPROVING THE QUALITY OF CIDER WITH THE HELP OF NATURAL ANTIOXIDANT AGENTS
(PhD Thesis Summary)

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Keywords: cider, plant extracts, polyphenols, flavonoids, antioxidant activity

INTRODUCTION

Innovation is currently a factor of particular importance in the desired and expected increase in cider consumption, a growing category of low-alcohol beverages, for which the consumer pays more than for a beer.

Phenolic compounds are responsible for the main organoleptic characteristics of most plants and also for their transformation products. They mainly contribute to color, bitterness and astringency in the case of drinks, the latter due to the presence of tannins. In addition, depending on their nature, they may have a nutritional and pharmacological interest, which determines the improvement of the quality of a drink such as cider, which pursues the doctoral thesis.

The main objective of the thesis is to contribute in an original way, through the presented studies, to the capitalization of some natural sources rich in antioxidant compounds, by incorporating them to the cider in the form of liquid extracts. All this, correlated with the use of ultrasound for the concentration of these compounds in the extracts or samples used, followed of course by an analysis of adjacent factors (processing conditions).

Achieving the main goal is of course preceded by the pursuit of secondary points, such as:

- to carry out a detailed literature review on this line,
- the establishment of steps for the production of cider under laboratory conditions, embodied in a technological flow chart,
- identification and evaluation of natural sources of antioxidants, with potential for their extraction,
- analysis of extracts obtained from prioritized sources based on preliminary results,
- evaluation of the ultrasound treatment, on the antioxidant content, and not only, on the cider enriched with blueberry and black carrot extracts, respectively
- performing statistical analyzes in the end (TPC, TFC, PPC and antioxidant capacity of cider variants).

The doctoral thesis is composed of two parts: the literature part and the original contributions.

PART I. LITERATURE DATA

The first part of the doctoral thesis cumulates a series of data collected from the literature, **starting from the acceptance of the term cider** with everything derived from it, from raw materials, nutrients and other ingredients on which the quality of the drink depends (additives, yeast cultures or enzymatic preparations), all detailed in the first chapter. In the second chapter, the comparative analysis of the technologies for obtaining cider was subsequently emphasized, finally concretizing a block scheme for obtaining it in laboratory conditions and last but not least the implications of using ultrasound for phenolic compounds. The third chapter focused on plant sources with antioxidant potential to improve the quality of cider, also studying the methods of separation from them in order to be capitalized.

Comparisons between the traditional method of cider production (using the microflora present on the skin of the fruit) and the methods of fermentation caused, led to the synthesis of yeasts and bacteria in fresh apple juice, which are listed in Table 1.11, from which it can be concluded that a well-defined yeast culture (pure strain) can be used to facilitate the fermentation process (present in this way in a sufficient concentration), the most widespread of them, being S. cerevisiae.

Table 1.11 *Typical microorganisms in freshly squeezed apple juice* (Jarvis B, 2003; Tascon N. F., Suarez Valles B.2007)

Type	**Species**	**Ability to increase to the acidity of apples (a)**	**Sensitivity to sulfites (b)**
Yeasts	Saccharomyces cerevisiae	++++	+-/-
	S. uvarum	++++	+-/-
	Saccharomycodes ludwigii	++++	-
	Kloeckera apiculata	++++	+++
	Candida mycoderma	++++c	++++
	Pichia spp.	++++c	++++
	Torulopsis famata	++++	++
	Aereobasidium pullulans	++++	+++
	Rhodoturola spp.	++++	++++
Bacteria	Acetobacter spp.	++++c	++
	Pseudomonas spp.	+	++++
	Escherichia colli	-/+	++++
	Salmonella spp.	-	++++
	Micrococcus spp.	+	++++
	Staphylococcus spp.	+	++++
	Bacillus spp.	-(celule)	-(spori)
	Clostridium spp.	-(celule)	-(spori)

a ++++ capable of good development, + capable of some growth, -/+ dependent on strains, - does not develop

b - insensitive, +/- relatively insensitive, ++,+++,++++ increasingly sensitive

c = only in the presence of air

This resulted in a series of consequences, bringing to the fore the need to use different forms of sulfur, correlated with the treatment of apple juice before fermentation and during the whole process, with an effect of course on color compounds (slowing down the coloring reactions etc.). Table 2.3 makes a recommendation regarding the amounts of sulfur required

for apple juice according to its pH, finally concretizing a diagram of the technological flow in case of beverages obtaining, for which the ultrasound usage represents an innovative point for the operations of phenolic compounds concentration "acceleration", with a role in the quality of cider.

Table 2.3 *Recommended sulfur concentration in apple juice at different pH values*

(Johansen K, 2012)

The pH of the Juice	**Sulfur concentration**
<3,0	0 ppm
3,0-3,3	75 ppm
3,3-3,5	100 ppm
3,5-3,8	150 ppm
3,8-4,0	200 ppm
>4,0	Malic acid at pH of 3.8 and addition of 150 ppm sulfur

Therefore, the release of phenolic compounds and anthocyanins, based on the use of ultrasound, applied in the practical part of the thesis on liquid extracts from plant sources with a high content in this regard, is the interest of the original contribution. Figure 3.7 summarized a series of methods for the separation, identification and isolation of such compounds (**polyphenols**), used mainly on a larger scale.

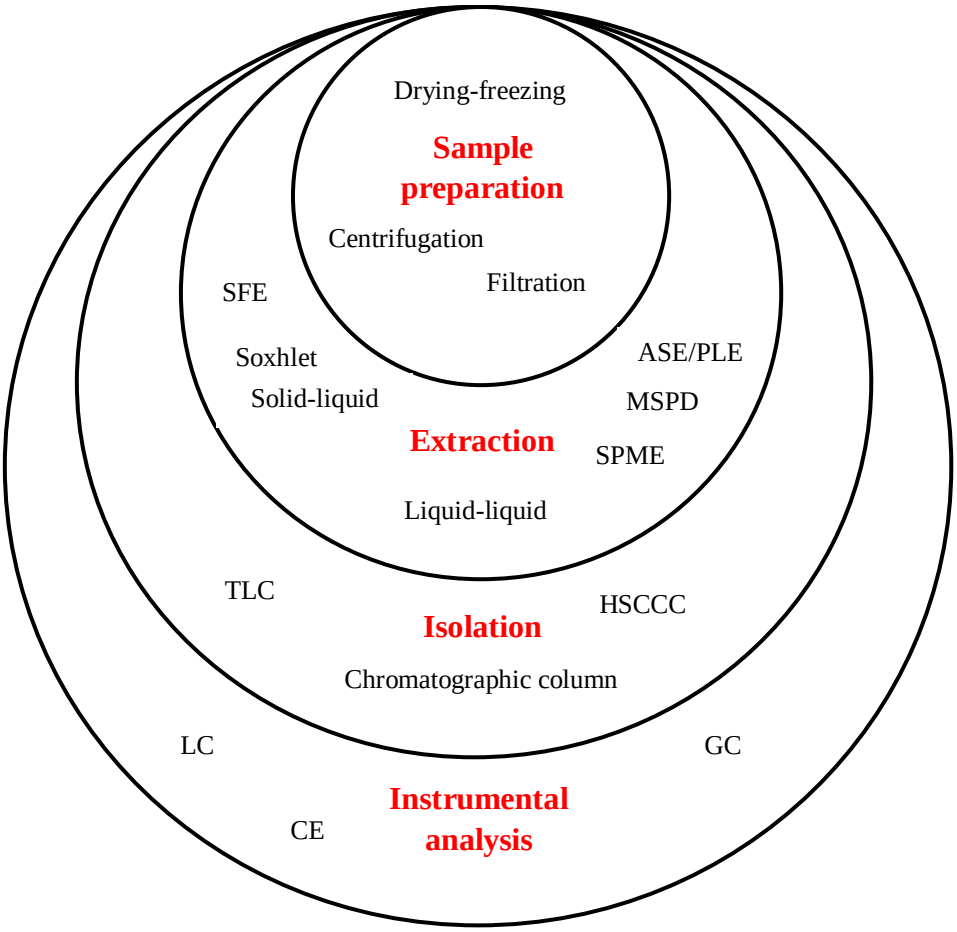


Fig. 3.7 *Methods used in the separation, identification and analysis of polyphenols* (Syed S. H. Rizvi, 2010)

Abbreviations: SFE-supercritical fluid extraction; SPME-solid phase microextraction; MSPD-matrix solid-phase dispersion; ASE/PLE-accelerated solvent extraction/pressurized solid lichid extraction; TLC-thin layer chromatography; HSCCC-high-speed counter-current chromatography; LC-electrochemical detector; GC-electron capture detection; CE-capillary electrophoresis

PART II ORIGINAL CONTRIBUTIONS

In the second part of the thesis, the first phase is the scientific research on obtaining the assortment of cider in laboratory conditions, taking into account the aspects mentioned above, as a starting point for carrying out the practical part of the research. Therefore, Chapter 4 reveals a number of aspects during the fermentation of apple juices, followed by a series of analyzes on the final cider.

Chapter 5 emphasizes the use of plant sources with antioxidant potential, in order to obtain liquid extracts, based on various solvents. Thus, the extraction conditions for each plant source that have been used are revealed, being divided into two main categories: aqueous media and ethanol-based media. Once the extraction scheme was finalized and applied to the chosen sources, they were tested to determine the content of polyphenols (TPC) and flavonoids (TFC), the best results being obtained in the case of blueberry spontaneous flora and H. sabdariffa in both cases. The measurements did not stop here, being estimated both the antioxidant activity of the obtained extracts, and theinterpretations of the influence of the solvent on each chosen plant source.

The cider variants subjected to ultrasonic tests, involved in the first phase the obtaining of blueberry extracts (culture, fresh state) using as solvent water with 2% citric acid, respectively those using a black carrot concentrate, and their enrichment. In Chapter 6, the influence of ultrasound on the increase of TPC and TFC from one measurement to another is presented for the samples to be tested.

Chapter 7 makes direct reference to the practical aspects during cider enrichment operations with these extracts (blueberries and black carrots), emphasizing that the experimental research of the study also focused on evaluating the effect of ultrasound treatment (different amplitudes, application times) on TPC, TFC, antioxidant power as well as on the evaluation of the antioxidant capacity and DPPPH activity of the analyzed ciders.

All this, followed in Chapter 8 by a series of statistical analyzes of the final results, including the precipitation capacity of the proteins being taken into account for the studied variants, and a series of conclusions in Chapter 9.

CHAPTER 4
OBTAINING CIDER IN LABORATORY CONDITIONS

4.1. Assortments of cider obtained from apples from the Voineasa area

The production of cider itself, for the experiments, involved the use of the fermentation method caused by the use of a yeast culture added to the "apple" must, simultaneously with a lot of practical aspects resulting from it (e.g. implications of metabisulphite usage). The yeast used to start the fermentation was of the genus Saccharomyces cerevisiae (see aspects during fermentation in Figure 4.2).



Fig. 4.2 *Aspects during the fermentation of apple juices (Brezan, 2018)*

Regarding the assortments of apples used to obtain cider, it was desired to highlight the Royal Gala variety, from Voineasa area, which leads to obtaining a cider with complex organoleptic characteristics (see malic acid content in the next subchapter).

4.2. Analyzes of cider varieties obtained in the laboratory

4.2.1. Determination of alcohol concentration using the ebuliometric method

4.2.1.1. Analayzed assortments

4.2.1.2. Method

4.2.1.3. Results

4.2.2. Determination of potential acidity of cider by potentiometric titration

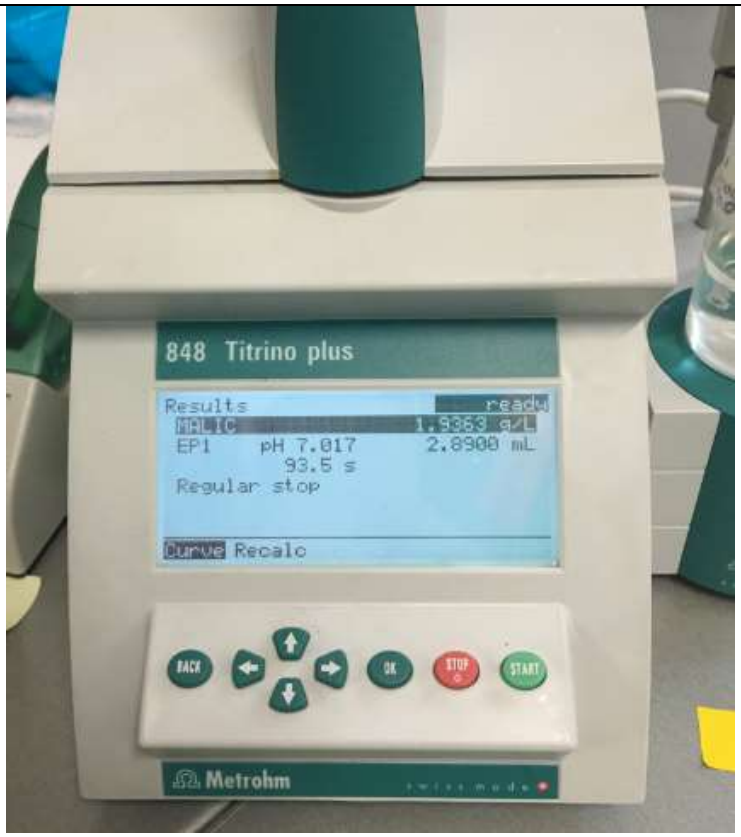
4.2.2.1. Analayzed assortments

It was desired to determine the potential acidity of cider, after measuring the alcoholic concentration (5.13% v / v alcohol by ebuliometric method) and to stop the fermentation by adding metabisulphite. The acidity expressed in g / L malic acid, confirmed the correlation of the value given in table 4.2, the inclusion of the apple variety used for cider in the category "sweet", as long as the titrated and analyzed cider sample, provided a value of less than 4.5 g / L malic acid.

4.2.2.2. Method

4.2.2.3. Results

Table 4.2 *Determination of cider acidity (Brezan, 2019)*

Titration method	**Acidity value**
Titration using **- distilled water -**	1.93 g/L 
Cider density [g/cm3] *	***0,99776***

4.2.3. Determination of sulfur dioxide by the RIPPER method

4.2.3.1. Analayzed assortments
4.2.3.2. Method
4.2.3.3. Results

In conclusion, the composition of fermented beverages, as well as other plant-derived products, is above all a function of the raw material. That is why a brief presentation of the main constituents (acids, tannins) in the plant material used for the development of fermented beverages and their location in the plant itself, is a point of interest because some of these compounds are extracted into the juice. A classification of apple varieties, used in the production of cider, both French and English, was established on the basis of the acidity and overall content of polyphenols (criterion << total tannins >>), reason for which it was also the subject of this study.

CHAPTER 5
OBTAINING AND TESTING PLANT EXTRACTS IN LABORATORY CONDITIONS

5.1. Materials and methods

5.1.1. Plant sources used

Of technological interest when we talk about the main polyphenols in apples and other plant materials are flavones, while when we talk about red pigments we are practically talking about anthocyanins. These components, useful for enriching cider from the "outside", are more or sometimes less easily extracted, depending on their solubility and the extraction environment of course, along with factors such as location within the plant. For these reasons, and due to the structural transformations they go through, they are correlated with intrinsic chemical reactions, which begin by segmenting the cellular integrity of plant material (such as crushing, chopping, etc. operations performed but not included in this summary) and continue during conservation process.

Table 5.1 reveals the plant sources of interest for the practical work, used for the enrichment of cider, in order to quantify and compare the content of polyphenols and flavonoids, in the first phase. These in addition to appreciating the antioxidant activity in the end.

Table 5.1. *Sources used to obtain extracts (Bădărău and Brezan, 2018)*

Plant source	Provenance	Variety / Region	Condition
cranberries	culture	Duke	fresh
	spontaneous flora	Prahova Valley	fresh
raspberry	culture	Polka	fresh
	spontaneous flora	Prahova Valley	fresh
blackberries	culture	Thornfree	fresh
	spontaneous flora	Prahova Valley	fresh
H.sabdariffa	trade	-	dry, powder
purple potato	culture	Purple blue Galanesti	fresh
		Blue Congo	fresh
		Blue de la Manche	fresh
		Patraque Auvergne	fresh

5.1.2. Extracts obtaining

In terms of solvents used, Table 5.2. shows both the variants that were tested and the notifications related to them.

Table 5.2. *Extraction conditions applied to plant sources (Bădărău and Brezan, 2018)*

Source	Provenance	Sample	Solvent			Time [h]
Cranberries	Spontaneous flora	1.1.	ETHANOL			24
		1.1. I	ETHANOL	CITRIC ACID	2%	24
		1.1 II	WATER			24
		1.1. III	WATER	CITRIC ACID	2%	24
		1.1. IV	WATER	VIT C	2ml	24
		1.1. V	WATER	CITRIC ACID	4%	24
Cranberries	Culture	1.2	ETHANOL			24

		1.2. I	ETHANOL	CITRIC ACID	2%	24
		1.2. II	WATER			24
		n/a	WATER	CITRIC ACID	2%	n/a
		n/a	WATER	VIT C	2ml	n/a
		n/a	WATER	CITRIC ACID	4%	n/a
Raspberry	Spontaneous flora	2.1.	ETHANOL			24
		2.1. I	ETHANOL	CITRIC ACID	2%	24
		2.1. II	WATER			24
		2.1. III	WATER	CITRIC ACID	2%	24
		2.1. IV	WATER	VIT C	2ml	24
		2.1. V	WATER	CITRIC ACID	4%	24
Raspberry	Cultură	2.2.	ETHANOL			24
		2.2. I	ETANOL	CITRIC ACID	2%	24
		2.2. II	WATER			24
		n/a	WATER	CITRIC ACID	2%	n/a
		n/a	WATER	VIT C	2ml	n/a
		n/a	WATER	CITRIC ACID	4%	n/a
Blackberries	Spontaneous flora	3.1.	ETHANOL			24
		3.1. I	ETHANOL	CITRIC ACID	2%	24
		3.1. II	WATER			24
		3.1. III	WATER	CITRIC ACID	2%	24
		3.1. IV	WATER	VIT C	2ml	24
		3.1. V	WATER	CITRIC ACID	4%	24
Blackberries	Cultură	3.2.	ETHANOL			24
		3.2. I	ETHANOL	CITRIC ACID	2%	24
		3.2. II	WATER			24
		3.2. III	WATER	CITRIC ACID	2%	24
		3.2. IV	WATER	VIT C	2ml	24
		3.2. V	WATER	CITRIC ACID	4%	24
Hibiscus	n/a	4	ETHANOL			24
		4 I	ETHANOL	CITRIC ACID	2%	24
		4 II	WATER			24
		4 III	WATER	CITRIC ACID	2%	24
		4 IV	WATER	VIT C	2ml	24
		n/a	WATER	CITRIC ACID	4%	n/a

The main steps followed in order to extract polyphenols and flavonoids are shown in figure 5.3.

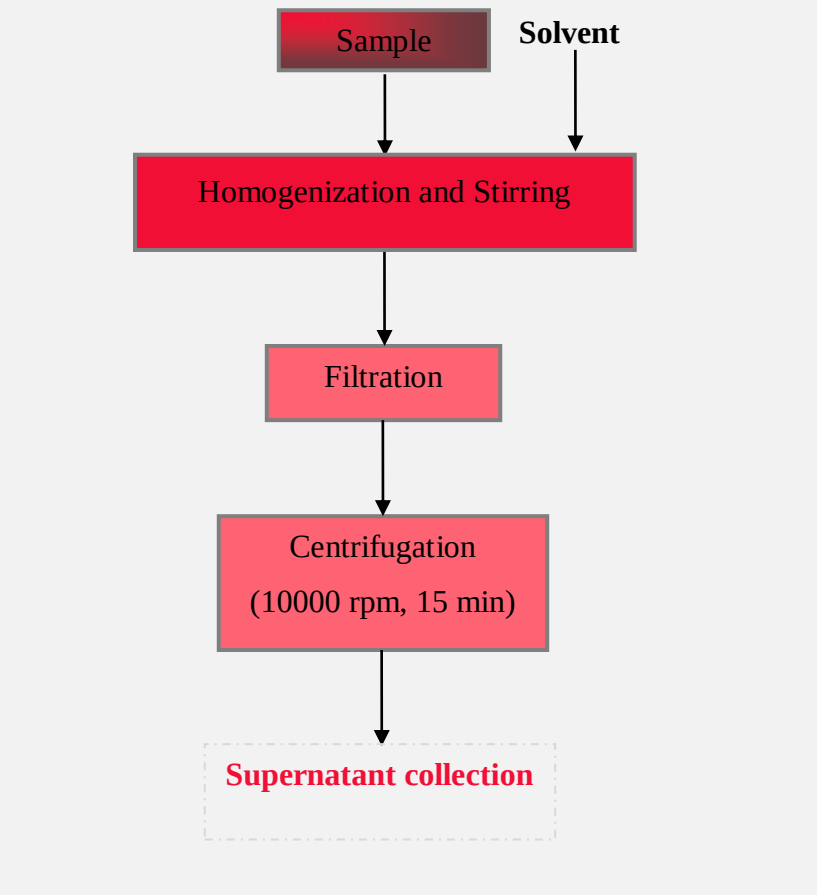


Fig. 5.3. *The main operations in the process of obtaining plant extract variants*

All these experiments were performed in three repetitions, finally the results being expressed as mean ± standard deviation.

5.2. Testing of the obtained extracts

5.2.1. Determination of polyphenol content in the extract (TPC)

5.2.1.1. Method

5.2.1.2. Results

Using the results obtained from the tests on each phenolic substrate involved in the discussion, for the extracts listed above, we developed a tabular comparison of the values obtained for each plant source tested, depending on the solvent used.

Therefore, Table 5.4. highlights that spontaneous flora blueberries provided the best values of polyphenol content among ethanol-based extracts, while H. sabdariffa delivered the best values for aqueous extracts.

Table 5.4. *TPC values vs. solvent used (Woinaroschy, Bădărău and Brezan, 2018)*

SOURCE	SOLVENT					
	Ethanol	**Ethanol CITRIC ACID 2%**	**Water**	**Water CITRIC ACID 2%**	**Water CITRIC ACID 4%**	**Water vit C**
Cranberry Spontaneous flora	829.529	1037.072	403.673	594.009	700.885	818.241
Cranberry Culture	682.754	945.905	358.140	-	-	-
Raspberry Spontaneous flora	804.048	922.518	289.737	411.141	497.501	823.402
Raspberry Culture	619.876	810.257	256.423	-	-	-
Blackberry Spontaneous flora	743.561	889.658	376.576	606.765	746.207	927.260
Blackberry Culture	663.222	762.504	323.176	486.219	588.237	872.541
Hibiscus	816.940	1012.183	531.429	839.290	-	885.204

5.2.2. Determination of flavonoid content in the extract (TFC)

5.2.2.1. Method

5.2.2.2. Results

On the other hand, the extraction of flavonoids, which are generally responsible for the characteristics of flavor, color or pharmacological activities, involved the use of the same types of extracts under the same conditions. Favorable results were of course obtained in the case of the same plant sources, as shown in Table 5.6. below, where synthetic TFC average values are presented.

Table 5.6. *TFC values vs. solvent used (Woinaroschy, Bădărău and Brezan, 2018)*

SOURCE	SOLVENT					
	Ethanol	**Ethanol CITRIC ACID 2%**	**Water**	**Water CITRIC ACID 2%**	**Water CITRIC ACID 4%**	**Water vit C**
Cranberry Spontaneous flora	33.440	34.455	13.554	12.688	16.592	11.608
Cranberry Culture	21.814	17.180	11.427			
Raspberry	15.356	9.294	7.697	8.386	4.657	6.459

Spontaneous flora						
Raspberry Culture	16.345	6.665	8.252			
Blackberry Spontaneous flora	34.171	15.863	10.169	12.669	10.167	10.678
Blackberry Culture	26.944	13.615	9.821	10.483	9.023	10.882
Hibiscus	86.856	26.548	24.593	41.807		43.033

5.2.3. Antioxidant properties

5.2.3.1. DPPH activity

5.2.3.2. Results

5.3. Discusions

It is obvious that in the case of the content of polyphenols in the extracts [TPC], significantly higher values were obtained compared to the other extracts, in the case of the test that used the mixture of ethanol with 2% citric acid as extraction medium. In this situation, the best results were delivered by blueberry spontaneous flora, as mentioned earlier, the value of the content in this source and using as a solvent, the mentioned mixture, being 1037.07 mgAGE / 100gr.

On the other hand, to determine the flavonoid content, good results were provided by ethanol-based extracts, used as such. As a major observation, in all cases, H. sabdariffa demonstrated the highest values in this regard.

5.3.1. Influence of the solvent for each plant source on the polyphenol / flavonoid content

Regarding the influence of the solvent on these already detailed contents, the application conditions were similar in terms of working temperature (20°C) or sample: solvent ratio (1:20). Comparisons of the [TPC] and [TFC] values regarding some experimental tests using distilled water as solvent, versus the solvent variants used in the previous extractions, are given suggestively in the following (Fig. 5.10, Fig. 5.11). No significant differences are observed, the content of biocompounds being relatively similar.

Table 5.8 *Average TPC and TFC values for extracts obtained using water as solvent (Woinaroschy, Bădărău and Brezan, 2018)*

	Plant source for obtaining the extract	TPC (mgAGE/100g)	TFC (mg QE/100g)
1	Blue purple Gălăneşti	**538.17**	**8.95**
2	Blue Congo	**549.61**	**7.45**
3	Blue de la Manche	**698.49**	**6.50**
4	Patraque Auvergne	**519.22**	**4.35**
5	Cranberry spontaneous flora	**956.98**	**112.02**
6	Cranberry culture	**1041.68**	**84.52**
7	Raspberry spontaneous flora	**716.41**	**9.35**
8	Raspberry culture	**732.58**	**6.13**

9	Blackberry spontaneous flora	**658.75**	**14.84**
10	Blackberry culture	**1088.61**	**16.56**
11	Hibiscus	**573.79**	**16.18**

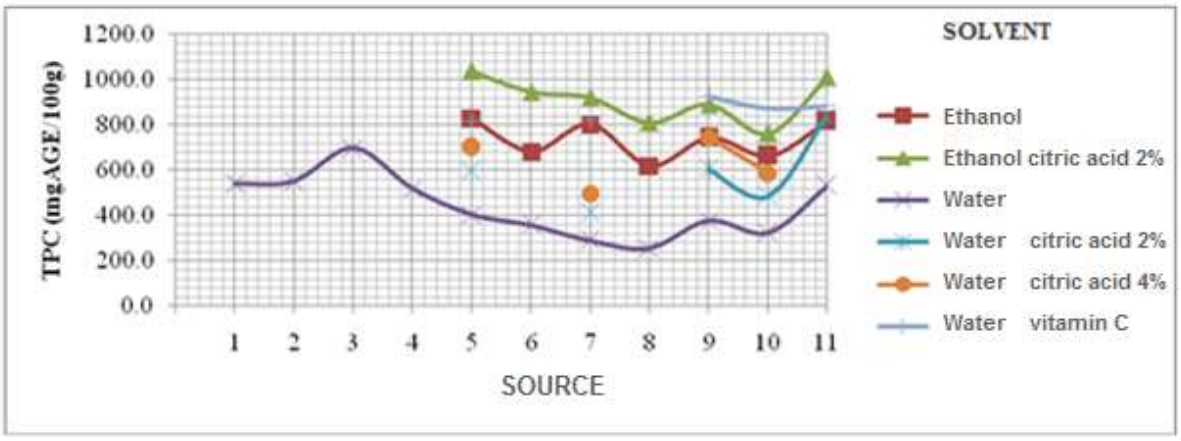


Fig. 5.10 *Average values of the total polyphenol content of the extracts according to the solvent and plant source used*

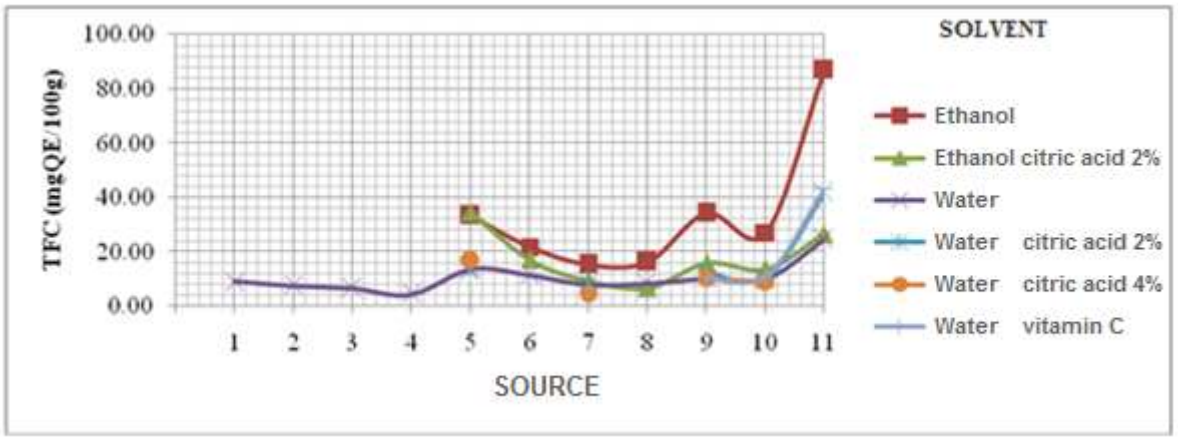


Fig. 5.11 *Influence of the solvent for each plant source on the flavonoid content*

CHAPTER 6
PRELIMINARY EXPERIMENTAL STUDY ON THE INFLUENCE OF PLANT ADDITIONS AND ULTRASOUND ON THE ANTIOXIDANT CAPACITY OF CIDER

6.1. Obtaining liquid extracts in laboratory conditions

6.1.1. Materials and methods

Considering the results obtained in the preliminary phase, it was used the blueberries, of the Duke variety and in the fresh state. Explanatory, the fresh samples (50 gr.) were ground over the amount of solvent that was initially dosed (200 ml) in order to homogenize in containers specially designed for this purpose. After a contact period of 24 h at a temperature of 20 ^{0}C (see table 6.2), they were subjected to the previous operations (stirring, filtration, centrifugation) to collect the extract itself.

6.1.2. Experimental variants

Using the cider previously obtained in laboratory conditions, it was desired to carry out the experimental part of the work, enriching it with a liquid extract concentrated in such antioxidant compounds, simultaneously with the application of ultrasound. Table 6.1 shows the experimental variants, coded according to the treatment applied and the plant source used to obtain the extract used.

Table 6.1 *Coding of experimental variants subjected to ultrasound treatments (Bădărău and Brezan, 2019)*

The plant source from which the extract was obtained		
Cranberries	**Black carrot extract**	**Amplitude [%]**
AM	MM	-
A1	M1	20
A2	M2	30
A3	M3	40
A4	M4	50

AM, MM – untreated controls (1/20 extract / cider mixtures)

6.2. Testing of the obtained extracts

Of course, the cider variants were analyzed in order to determine the content of polyphenols, respectively flavonoids. In the case of polyphenol content in the extract (TPC), the average of the values read for the absorbance of the samples at wavelengths of 725 nm and the values (TPC) as shown in Figure 6.1 were summarized, these being expressed in mgAGE / 100gr and representing the point starting for the graphical representation below (while only the solvent is changed).

6.2.1. Determination of polyphenol content in the extract (TPC)

6.2.1.1. Method

6.2.1.2. Results

Figure 6.1 shows that high values of ultrasound amplitude also provide higher values of the total polyphenol content in the tested variants.

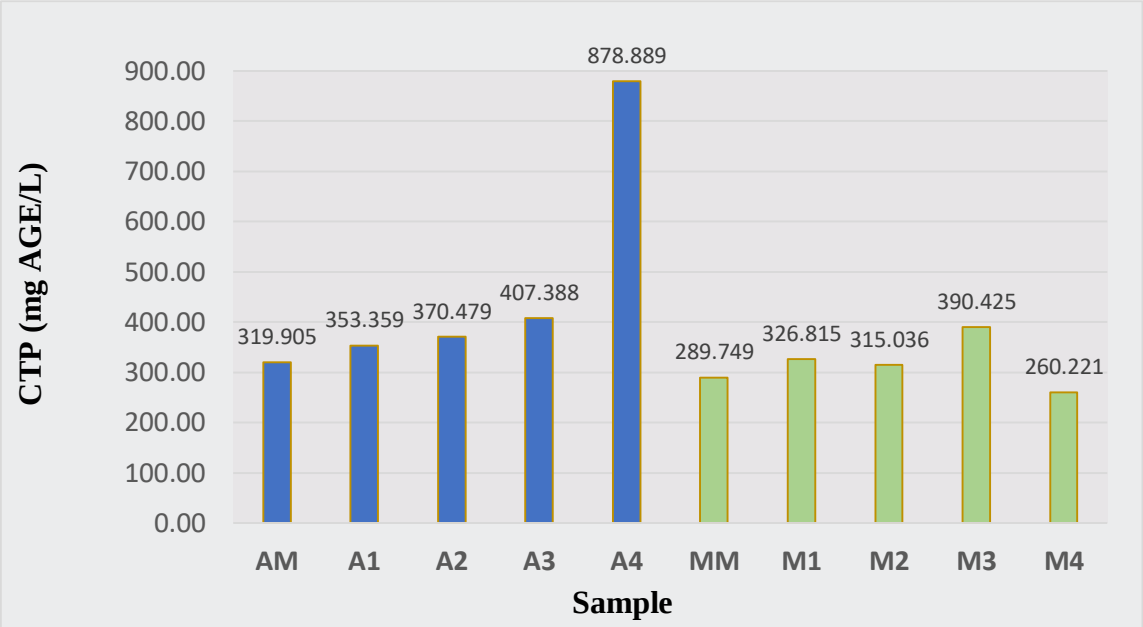


Fig. 6.1 *Mean TPC values for experimental cider variants obtained with cranberry extracts [2% citric acid] and ultrasound-treated black carrot extracts*

6.2.2. Determination of flavonoid content in the extract (TFC)

6.2.2.1. Method

6.2.2.2. Results

On the other hand, for the determination of the flavonoid content (TFC) the values for the absorbance of the samples at wavelength of 510 nm were read, in figure 6.2 being found the values (TFC) for the analyzed ciders.

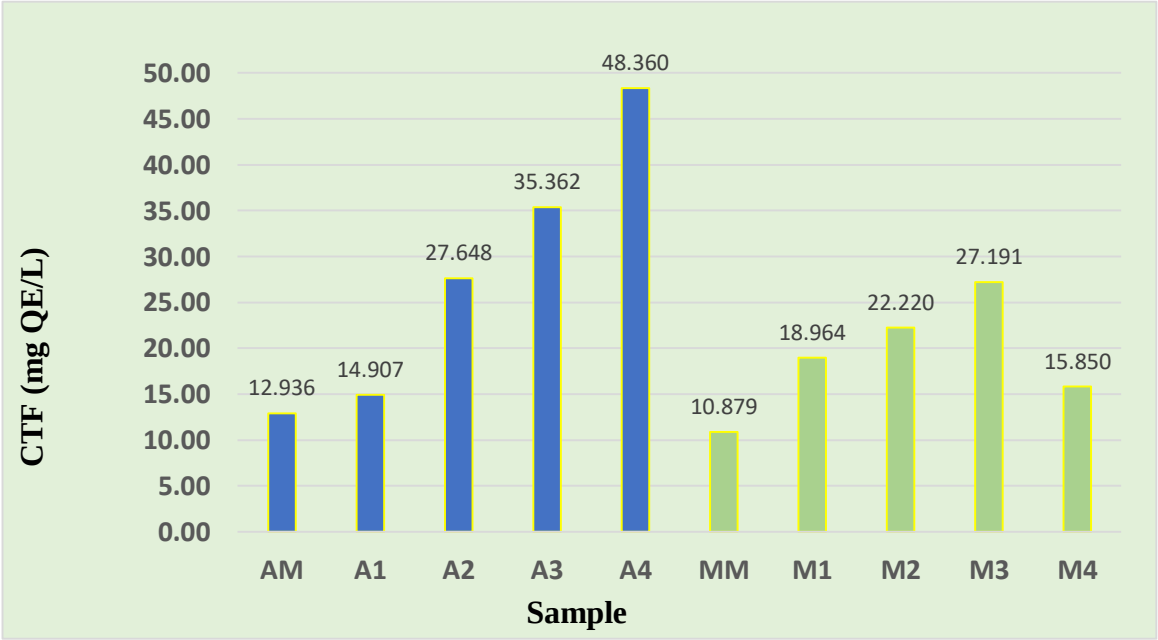


Fig. 6.2 *Average TFC values for experimental cider variants obtained with blueberry extracts [2% citric acid] and ultrasound-treated black carrot extracts*

6.3. Antioxidant properties

6.3.1. DPPH activity

6.3.1.1. Results

6.4. Discusions

6.4.1. Influence of amplitude on sample temperature

There is an increase in values for both TPC and TFC, from one measurement to another, discussing here each of the ciders enriched with both types of extracts. Therefore, we can note a first effect of the use of ultrasound. These results are due to the change (increase) of the amplitude of the ultrasound in our propagation medium (cider + liquid extract).
Concluding on the use of ultrasound, we can say that the best values of these contents were obtained by subjecting the samples to an amplitude of 50% of them.

Discussing the influence of amplitude on the sample temperature, we can see in the following figure the resulting temperatures at the end of cider ultrasound, being easy to conclude that with increasing amplitude there is an increase in ultrasound temperature.

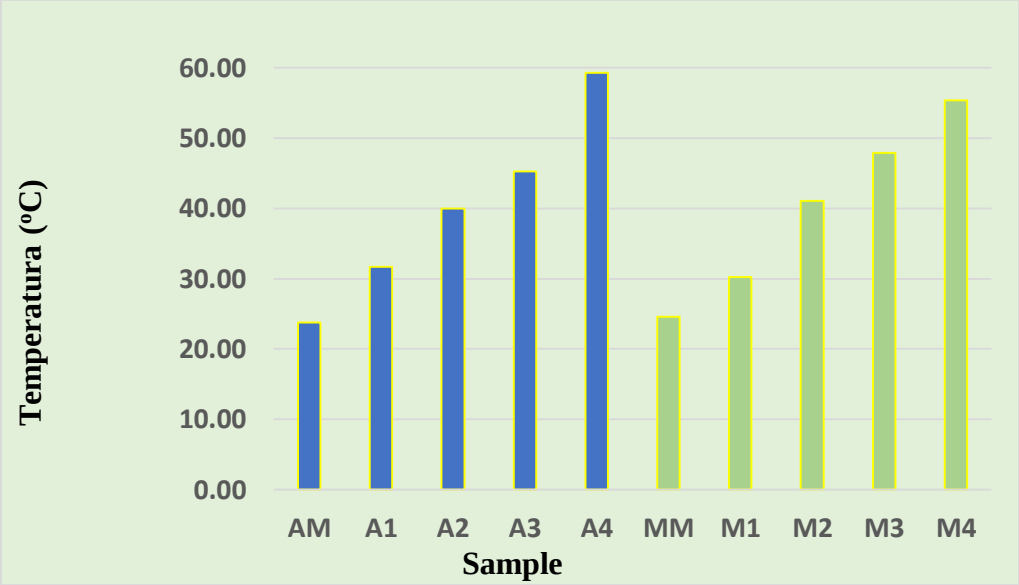


Fig. 6.5 *Temperature changes during the ultrasound of cider samples with the addition of plant extracts*

6.4.2. Influence of treatments on the acidity, pH of the samples

CHAPTER 7
PRACTICAL ASPECTS REGARDING THE ENRICHMENT OF CIDER WITH BLUEBERRY AND BLACK CARROT EXTRACTS

7.1. Introdution

The focus of the thesis is to highlight the effect of tested extracts and of course ultrasound, on TPC, TFC, antioxidant properties and the ability of proteins to precipitate from these cider variants. The enrichment of the polyphenol content in cider obtained in laboratory conditions (these have an overwhelming importance from a sensory point of view, with implications on its preservation capacity) is the subject of the practical part, the study wishing to capitalize on some plant sources and the benefits of using ultrasound with this purpose.

Over time, ultrasonic assisted extraction as well as its opportunities in the food processing industry have proven a number of advantages, due to its classification as a non-thermal processing method, hence many benefits resulting (e.g. minimizing processing or protecting safety food). These were some considerations taken into account, before their involvement in the realization of the practical part of the reasearch, the experimental studies focusing on the following aspects:

- evaluation of the effect of ultrasound treatment (different amplitudes and application times) on TPC, TFC, antioxidant power and protein precipitation capacity, on cider enriched with black carrot and blueberry extracts;
- evaluation of the antioxidant capacity of cider variants, using: FRAP test (Ferric Reducing Antioxidant Power), ABTS test (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH activity (diphenylpicrylhydrazyl).

Last but not least, the precipitation capacity of proteins was also considered important, as the tendency of polyphenolic compounds in cider is to form solid compounds containing proteins (formation of turbidity in beverages mainly).

7.2. Materials and methods

7.2.1. Chemicals and reagents

7.2.2. Obtaining cider and fermentation process

Obtaining apple wort from the Royal Gala variety was preceded by the use of vitamin C, a compound that can have a degrading effect on the yeast naturally present in the wort, and therefore also delays the fermentation process.
In order to start the fermentation of the apple must collected in glass jars, an exogenous yeast, the genus Saccharomyces cerevisiae, was added. The safety of starting the fermentation was also taken into account since the use of the adjuvant mentioned above, could compromise the activity of the indigenous microflora.

Stopping the fermentation carried out at a temperature of 19 ^{0}C, when the alcoholic concentration reached 5% alc./vol (5.13% vv) was desirable, the cider being treated with sulfur dioxide and subjected to the maturation operation (storage at refrigeration temperature). The amount of sulfur used in each decantation was 50 ppm, not exceeding a total of 200 ppm by summarizing all additions.

7.2.3. Extracts added to cider

As mentioned, two extracts were used to improve the antioxidant capacity of cider, as shown in the explanatory tables in previous chapters:

- **black carrot dye (ready-to-use extract, commercial in nature, 0.3% added, v / v),**
- **blueberry extract (obtained in laboratory conditions and added to cider variants in a proportion of 5% v / v).**

As already known, both extracts were added before ultrasound treatment.



Fig. 7.1 *Aspects during the addition of cider extracts (Badarau and Brezan, 2019)*

7.2.4. Ultrasound treatment

The results presented reflect the values for cider variants enriched with ultrasound-treated extracts (VCX-750, Sonics & Materials, Inc.Newtown, CT, USA) at 750 W, with a constant frequency of 20 kHz, at 20%, 30% and 40% amplitude for 5 minutes, using a 19 mm probe and a 500 ml sample of cider. This combination of amplitude and time was established to make a comparison of the release of antioxidant compounds under different conditions. We used low values for amplitude and time, in order not to improve the values of the protein precipitation capacity.

7.2.5. Sample analysis

CHAPTER 8
STATISTICAL ANALYSIS OF RESULTS

8.1. Cider variants and classification

In order to statistically analyze the results of the tests performed on the cider variants presented in table 8.1, the software SPSS (Statistical Package for the Social Sciences) was used, being analyzed by ANOVA and Duncan's Multiple Range Test. Regarding the correlation between the variables, it was also analyzed using Pearson correlation coefficients. The experimental data on the addition of extracts, respectively amplitudes and application times of ultrasound, are presented below:

Table 8.1 *Coding (ID) of cider variants obtained using the addition of extracts and ultrasonic treatment (Bădărău and Brezan, 2020)*

Extracts added	Amplitude (%)	Time (min)	ID Cider variants
Black carrot extracts (0.3%, v/v)	-	-	C
	A 20%	2	C202
		5	C205
		7	C207
	A30%	2	C302
		5	C305
		7	C307
	A40%	2	C402
		5	C405
		7	C407
Blueberry extracts (5%, v/v)	-	-	B
	A 20%	2	B202
		5	B205
		7	B207
	A30%	2	B302
		5	B305
		7	B307
	A40%	2	B402
		5	B405
		7	B407
Cider without added extract			M

8.2. Total polyphenol content (TPC)

Table 8.2 reveals that samples that underwent ultrasound treatments at lower amplitude values reported higher TPC values. The good thing is that ultrasound can improve the release of these compounds found in vacuole-soluble form or bound to pectin from the cell wall through the cavitation phenomenon.

As can be seen from the data below, the highest value of TPC for the analyzed ciders was obtained using the amplitude A 20% and 5 minutes as application time, in both variants, both enriching with black carrot and with blueberries.

Table 8.2 Total Polyphenol content of the tested cider variants (Bădărău şi Brezan 2020)

	Code	TPC (mg GAE/L)[1]	±	SD	*Duncan*[2]
Black carrot extracts and ultrasound treatment	C202	503.8	±	101.9	*b*
	C205	730.5	±	38.6	*a*
	C207	423.6	±	51.2	*bc*
	C302	303.6	±	35.4	*d-g*
	C305	411.0	±	84.8	*c*
	C307	294.1	±	91.5	*d-g*
	C402	287.9	±	68.0	*d-G*
	C405	339.3	±	36.6	*cde*
	C407	208.5	±	38.2	*fghi*
Cranberry extracts and ultrasound treatment	B202	279.5	±	25.0	*d-h*
	B205	415.6	±	21.4	*bc*
	B207	167.6	±	39.2	*i*
	B302	296.5	±	30.1	*d-g*
	B305	312.8	±	23.9	*d-e*
	B307	155.5	±	95.7	*i*
	B402	174.3	±	38.5	*i*
	B405	184.0	±	28.8	*hi*
	B407	152.5	±	18.0	*i*
" Control " type tests untreated	M Cider	191.1	±	32.7	*ghi*
	C	368.3	±	24.7	*cd*
	B	242.3	±	21.9	*e-i*

1Data refer to ± SD of a number of 3 experiments (n = 3) .2Values with different letters differ significantly from the ANOVA and Duncan tests (P <0.05). Abbreviations: TPC = Total Polyphenol Content; GAE = Gallic Acid Equivalent; SD = Standard Deviation

Therefore, a proven effectiveness of ultrasound treatments is observed, and not only by improving the recovery of the compound yield, but also helps to reduce the extraction time, resulting in economic benefits.

8.3. Total flavonoid content (TFC)

Of course, in the case of flavonoid content, the ultrasound-treated samples showed higher values compared to the "Control" type samples. Referring to control sample C (30.42 ± 3.154 mg QE / L) for example, for black carrot-enriched cider, we have significantly better values, which were provided based on ultrasound treatment at an amplitude of 20% (43, 82 ± 7.843 mg QE / L for 2 minutes, 45.13 ± 1.655 mg QE / L for 5 minutes and 40.62 ± 1.450 mg QE / L for 5 minutes, respectively), as shown in the following table.

Table 8.4 *Total Flavonoid content of the tested cider variants*
(Bădărău and Brezan, 2020)

	Code	TFC (mg QE/L) [1]	±	SD	*Duncan*[2]
Black carrot extracts and ultrasound treatment	C202	43.82	±	7.843	*a*
	C205	45.13	±	1.655	*a*
	C207	40.62	±	1.450	*a*
	C302	28.05	±	2.437	*cd*
	C305	31.28	±	3.776	*bc*
	C307	18.76	±	1.490	*ef*
	C402	12.45	±	1.022	*gh*
	C405	11.45	±	1.442	*h*
	C407	12.02	±	2.853	*h*
Cranberry extract and ultrasound treatment	B202	31.99	±	3.387	*bc*
	B205	35.85	±	1.618	*b*
	B207	15.82	±	0.535	*eh*
	B302	13.34	±	2.400	*gh*
	B305	19.62	±	2.664	*e*
	B307	14.45	±	0.907	*fgh*
	B402	15.28	±	2.520	*e-h*
	B405	11.68	±	1.543	*h*
	B407	11.45	±	1.442	*h*
" Control " type tests untreated	M Cider	17.42	±	0.858	*e-g*
	C	30.42	±	3.514	*c*
	B	25.68	±	1.371	*d*

1Data refer to ± SD of a number of 3 experiments (n = 3) .2Values with different letters differ significantly from the ANOVA and Duncan tests (P <0.05). Abbreviations: TPC = Total Polyphenol Content; GAE = Gallic Acid Equivalent; SD = Standard Deviation

8.4. Protein precipitation capacity (PPC)

For the purpose of this research, regarding the determination of the PPC value, the absorbance values at 510 nm were taken into account. Table 8.6 shows the values of this parameter, which are correlated with the TPC and TFC values. In conclusion, the cider variants that provided the highest TPC and TFC values also showed better values in the case of PPC due to the treatments applied.

These results, however, contradict the theory that the interaction between polyphenols and proteins is the most common cause of turbidity in beverages. Usually, procyanidins (flavonols) are considered the main contributors to the formation of turbidity, but cider variants with high TFC values have also demonstrated a good precipitation capacity of proteins, these mechanisms not being investigated.

Table 8.6 *Protein Precipitation Capacity for cider variants with the addition of extracts and ultrasound treatments*
(Bădărău and Brezan, 2020)

	Code	PPC (A510nm) [1]	±	SD	*Duncan*[2]
Black carrot extracts and ultrasound	C202	1.033	±	0.074	*b*

treatment	C205	1.404	±	0.054	*a*
	C207	1.089	±	0.128	*b*
	C302	1.129	±	0.134	*b*
	C305	1.033	±	0.074	*b*
	C307	1.077	±	0.226	*b*
	C402	0.510	±	0.130	*def*
	C405	0.587	±	0.074	*c-f*
	C407	0.440	±	0.044	*fg*
Cranberry extract and ultrasound treatment	B202	0.677	±	0.038	*cd*
	B205	0.698	±	0.136	*c*
	B207	0.659	±	0.039	*cde*
	B302	0.491	±	0.041	*e-g*
	B305	0.560	±	0.120	*c-f*
	B307	0.246	±	0.083	*h*
	B402	0.326	±	0.058	*gh*
	B405	0.262	±	0.052	*h*
	B407	0.184	±	0.060	*h*
" Control " type tests untreated	M Cider	0.736	±	0.120	*c*
	C	0.959	±	0.039	*b*
	B	0.660	±	0.115	*efg*
LSD 5%0.148		LSD 1%0.199		LSD 0.1%	0.262

1Data refer to ± SD of a number of 3 experiments (n = 3) .2Values with different letters differ significantly from the ANOVA and Duncan tests (P <0.05). Abbreviations: PPC = Protein Precipitation Capacity

8.5. Antioxidant capacity of cider variants

Table 8.7 summarizes the antioxidant capacity of the experimental cider variants, evaluated using the FRAP, ABTS and DPPH tests. As shown in this table, the inhibition values of FRAP, ABTS and DPPH were significantly different between the ciders tested (p <a0.05). And this time, to the cider which was added black carrot being subsequently treated with ultrasound at an amplitude of 20% for 5 min, is assigned the highest values in terms of antioxidant capacity expressed by all tests (14.69 ± 0.09 Trolox mmol / L for FRAP test, 25.79 ± 0.89 Trolox mmol / L for ABTS test, respectively 65.35 ± 2.59% for DPPH radical inhibition%). At the opposite end are the cider variants (with blueberry extracts) treated at the highest aptitude and time period (B407), (2.16 ± 0.18 Troloxmmol / L for FRAP test, 5.48 ± 1, 75 Trolox mmol / L for the ABTS test, respectively 25.94 ± 4.309% for inhibition of DPH radicals).

Table 8.7 *Antioxidant capacity of experimental cider variants, enriched with extracts and ultrasound treated, expressed by FRAP, ABTS and DPPH inhibition values* (Bădărău and Brezan, 2020)

ID	**FRAP (Trolox mmol/L)**	**±SD**	***Duncan***	**ABTS (Trolox mmol/L)**	**±SD**	***Duncan***	**DPPH %inh**	**±SD**	***Duncan***
C202	11.02	±0.33	*b*	19.13	±0.70	*cd*	61.90	±3.570	*ab*
C205	14.69	±0.09	*a*	25.79	±0.89	*a*	65.35	±2.587	*a*
C207	9.76	±0.09	*d*	10.58	±0.05	*gh*	53.94	±7.377	*b-e*
C302	10.23	±0.34	*c*	17.45	±0.93	*e*	55.44	±6.648	*a-d*
C305	7.75	±0.18	*f*	21.57	±1.06	*b*	59.28	±1.108	*a-c*

C307	4.02	±0.23	*ij*	15.84	±1.13	*e*	43.43	±5.561	*efgh*
C402	3.10	±0.12	*m*	9.57	±0.58	*gh*	46.41	±2.453	*d-g*
C405	3.37	±0.19	*lm*	7.60	±0.89	*i*	44.99	±5.685	*d-g*
C407	2.51	±0.09	*n*	7.40	±0.49	*i*	37.24	±6.416	*fghi*
B202	8.52	±0.23	*e*	13.36	±0.52	*f*	51.10	±8.628	*a-d*
B205	9.44	±0.19	*d*	18.41	±1.48	*cd*	56.29	±2.884	*b-e*
B207	5.63	±0.26	*g*	10.96	±0.82	*gh*	45.34	±3.208	*d-g*
B302	3.80	±0.34	*jk*	7.40	±0.49	*ij*	45.91	±4.309	*d-g*
B305	4.43	±0.23	*hi*	9.09	±0.65	*h*	48.40	±1.398	*c-f*
B307	3.57	±0.04	*kl*	5.80	±0.79	*j*	29.21	±7.221	*ij*
B402	3.16	±0.27	*m*	6.48	±0.60	*ij*	23.67	±5.685	*j*
B405	2.34	±0.07	*n*	7.64	±0.22	*i*	29.50	±4.560	*ij*
B407	2.16	±0.18	*n*	5.48	±1.75	*j*	25.94	±4.309	*j*
M	3.28	±0.10	*lm*	6.60	±0.32	*ij*	26.58	±8.805	*ij*
C	5.91	±0.16	*g*	14.88	±0.84	*e*	46.23	±3.375	*d-g*
B	4.31	±0.14	*hi*	9.57	±0.58	*gh*	36.60	±1.303	*ghi*
LSD 5%		0.345		1.431			8.542		

1Data refer to ± SD of a number of 3 experiments (n = 3). 2Values with different letters differ significantly from the ANOVA and Duncan tests (P <0.05). Abbreviations: (DPPH = Diphenyl picrylhydrazyl, FRAP = Ferric Reducing Antioxidant Power, ABTS = 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)).

Our studies show higher values of antioxidant capacity reported by Picinelli (2009). Compared to other alcoholic and non-alcoholic beverages, the antioxidant power of many of these cider variants was significantly higher.

In the present study, TPC and TFC values were correlated with antioxidant activity, estimated using FRAP, ABTS and DPPH% inhibition tests. The lowest value of the correlation coefficient (r2) was obtained in the case of DPPH. Our study shows that FRAP test values were correlated with TFC values (r2 = 0.873). In all cases, the best correlations were the identification of TFC values compared to TPC (see table 8.9).

Table 8.9 *Pearson correlation coefficients for the interaction of TPC, TFC, PPC and antioxidant capacity of cider variants (expressed by ABTS, FRAP tests and DPPH inhibition values%)*

(Bădărău and Brezan, 2020)

	TFC (mg QE/L)	**FRAP (Troloxmmol/L)**	**ABTS (Troloxmmol/L)**	**DPPH (% inhibare)**	**PPC (A 510nm)**
TPC (mg GAE/L)	0.795**	0.798**	0.766**	0.746**	0.759**
TFC (mg QE/L)		0.934**	0.795**	0.753**	0.763**
FRAP (Trolox mmol/L)			0.849**	0.787**	0.817**
ABTS (Trolox mmol/L)				0.778**	0.839**
DPPH (% inhibare)					0.776**
Sig. (2-tailed)	.000	.000	.000	.000	.000
N	54	54	54	54	54

** The correlation is significant at the level of 0.01 (2-tailed)

Regarding the DPPH test, % of radical inhibition of DPPH by antioxidants was attributed to their ability to donate hydrogen. Figure 8.2 shows the scavenging activities for DPPH radicals, the inhibition of DPPH free radicals increasing from 37.24 ± 6.416% to 65.9 ± 2.587% for cider enriched with black carrot extracts and from 25.94 ± 4.309% to 56.29 ± 2.884% for variants with blueberry extracts (Woinaroschy, Bădărău and Brezan, 2020).

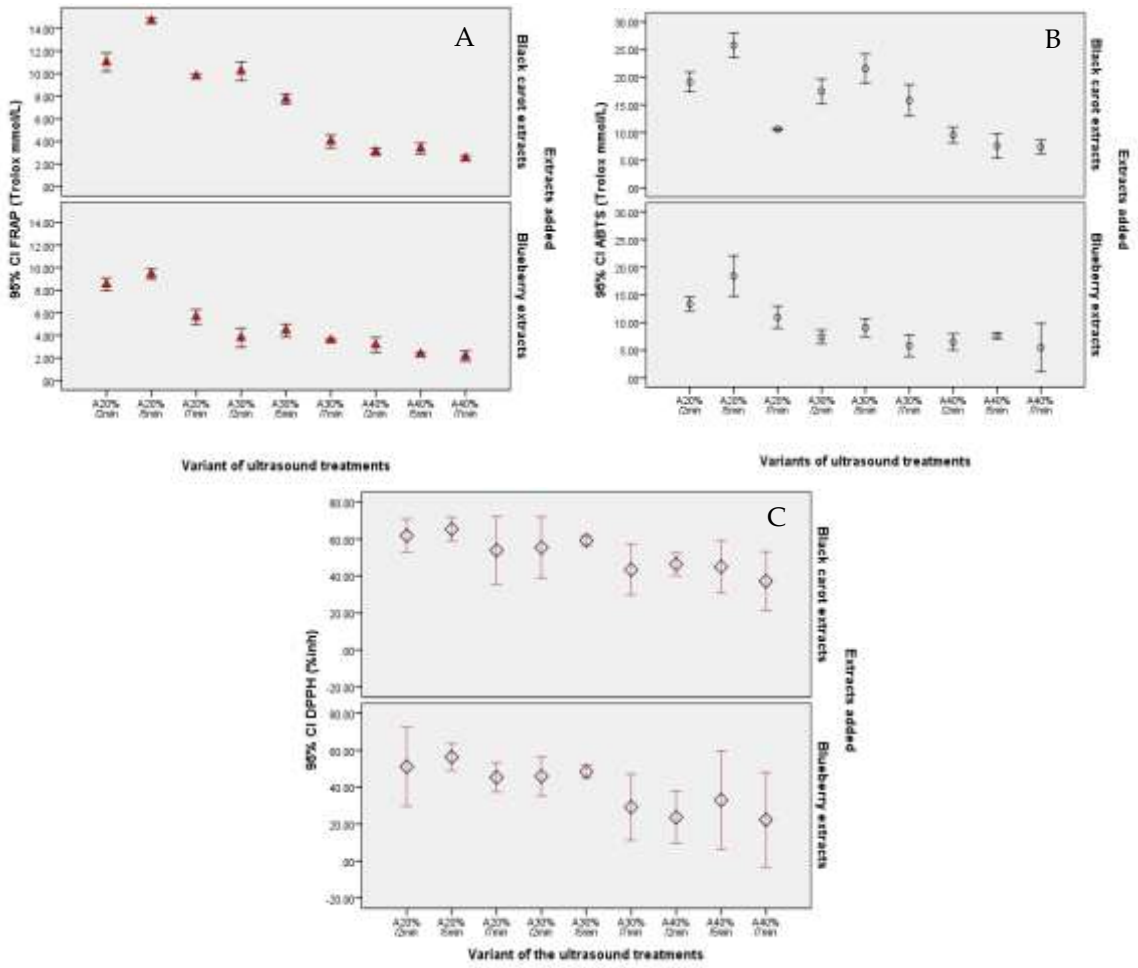


Fig.8.2 *The effects of ultrasound treatment and the addition of extracts (to obtain antioxidant-rich cider) on the results of the FRAP test (Troloxmmol / L) (A), the results of the ABTS test (Troloxmmol / L) (B), and on the inhibition of DPPH% (C) for the cider variants obtained*

As shown in Figure 8.2 A&B, the ultrasound conditions for cider with added extracts had a significant influence on the antioxidant activity tested by FRAP and ABTS.

Good results of antioxidant capacity were shown using a lower amplitude (in our case A 20%) and a period of time of 5 minutes applicability, which is probably due to the high sensitivity of polyphenol compounds to higher amplitudes of ultrasound (Woinaroschy and Brezan, 2020). The observation is aligned with some previous findings made by Nayak and Rastogi (2011), which show that an amplitude of 10 ... 14% was optimal for improving antioxidant activity, the conclusions leading to go further in our experiments to optimize the process.

CHAPTER 9
GENERAL CONCLUSIONS AND OPENINGS OF THE DOCTORAL THESIS

9.1. General conclusions

The doctoral thesis entitled "Improving the quality of cider with the help of natural antioxidants agents" is the result of research conducted at the Polytechnic University of Bucharest, Faculty of Applied Chemistry and Materials Science, Department of Chemical and Biochemical Engineering, and at the National Institute of Research Development for Potato and Sugar Beet - INCDCSZ Braşov.

The studies that are presented aimed at capitalizing on plant sources rich in antioxidants by incorporating them into cider, in the form of liquid extracts obtained in the laboratory, and the effects on improving the content of polyphenols and flavonoids. In order to carry out the experiments, ultrasound was also involved, with implications in increasing the content of such compounds in the tested cider variants.

In order to achieve the main objective of the work, namely to make original contributions to the enrichment of cider with antioxidant compounds of an exogenous nature, in order to improve its quality, the work also benefits from the use of ultrasound, which can be widely used in the food industry, adding value to the research undertaken.

For these reasons, it was necessary to draw some secondary objectives in order to achieve the main objective of the thesis, namely:

- study on the existing data in the specialized literature regarding the acceptance of the term cider, particularities of the raw material, obtaining technologies, etc.,
- laying down the steps for obtaining cider under laboratory conditions, embodied in a technological flow chart,
- research on the use of ultrasound in food, with direct reference to cider,
- obtaining liquid extracts in laboratory conditions, on various media and from different plant sources (blueberries, raspberries, blackberries and H. Sabdariffa flowers) rich in antioxidants,
- prioritization of obtaining and testing extracts (blueberries, respectively black carrots), with subsequent addition to cider, based on initial results,
- capitalization of the use of ultrasound at various amplitudes, in order to increase the TPC and TFC values from the tested cider variants,
- carrying out a statistical analysis of the results obtained on this topic.

Following experimental research, both the level of total polyphenols and the content of flavonoids, correlated with the antioxidant capacity (FRAP, ABTS and DPPH testing) for several enriched cider variants by adding black carrot and blueberry extract respectively, and subsequently treated by ultrasound, were highlighted. The implications of ultrasound in increasing the content of such compounds were also highlighted in this way.

The evaluation of the impact of ultrasound on antioxidant compounds was done by using them at different amplitudes and each time other times of application of ultrasound samples. The target compounds were detected in significant quantities in the treated variants using a frequency of 20 kHz, at an amplitude of 20% and for a period of applicability of 5 minutes in the case of all determinations.

The studies also incorporate the side effects of the use of ultrasound (such as influences on the temperature of the samples), wishing to be a study as complex as possible. Precisely for this reason, the antioxidant activity of ultrasound samples is implicitly revealed.

9.2. Openings of the doctoral thesis

The studies denote the value of fundamental and also applied research, the results obtained from experimental research can be reference elements for potential uses of plant sources, in strengthening the meaning of functional food in many foods and not just cider.

The approach, not only allowed to reach several sub-points, but also convinced of some openness in the field of obtaining and using such liquid extracts, rich in antioxidant compounds. Here we can list among the most important, testing the antioxidant power of anthocyanin pigments from plant sources used and the influence of the solvent on the content extracted from these sources, along with the application of ultrasound at different amplitudes, to increase antioxidant content and side effects.

SCIENTIFIC WORKS DEVELOPED DURING THE COURSE DOCTORAL INTERNSHIP

During the doctoral internship, the results of scientific research were published in journals and expressed in communications of scientific papers as follows:

A. Articles in ISI indexed journals:

1. Effects of blueberry and black carrot extracts addition on antioxidant properties and protein-precipitating capacity of ultrasound-treated cider, Boris Brezan, Carmen Bădărău, Alexandru Woinaroschy, **Processes, 2020, 8, 812; doi:10.3390/pr8070812 - 10 July 2020 , ISI Factor de impact: 2,753;**
2. Effects of ultrasound treatments on antioxidant content of cider, enriched previously with natural extracts, Boris Brezan, Carmen Bădărău, Aleandru Woinaroschy, Vasile Padureanu, **Revista de Chimie, 2020, 71(4), 263-268, ISI Factor de impact: 1,755. (Articol trimis in data 10.11.2019)**
3. The total content of polyphenols and flavonoids of some plants extracts, as potential additions for cider obtaining, Alexandru Woinaroschy, Carmen Bădărău, Boris Brezan, **U.P.B. Sci. Bull., Series B, 81, 4, 23-30, 2019.**

B. International Conferences:

1. Usage of ultrasound treatment in case of experimental variants of cider, 21th Romanian International Conference on Chemistry and Chemical Engineering – RICCCE 2019, Constanta 4-7 Septembrie 2019, Boris Brezan, Alexandru Woinaroschy, Carmen Bădărău.

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