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TITLE

INVESTIGATION DICARBONYL COMPOUNDS GENERATED FROM THE MAILLARD REACTIONS OF METHIONINE WITH REDUCING SUGARS TO ENFOLD OFF FLAVOR IN GOAT'S MILK

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ABSTRACT

Population of goat in Indonesia remains low and stagnant (about 1.2 % per year). In the other hand, Indonesia is a tropical country, which is suite for the goat's growth. One of strong reason is low in goat-based product's demand since "goaty" flavor existed in goat milk is off-flavor that is highly responsible for the consumer dislike. Therefore, to increase the demand, the reduction of "goaty" flavor is suggested. Our previous research showed the strategy to reduce intensity of "goaty" flavor by folding off-flavor with maillard product. Rare sugar is the new substances beside having zero calories and high antioxidant, but also acting as amino-binding in maillard reaction. Among rare sugars, only three: fructose, psicose, and tagatose that able to produce high intensity of intermediate product from maillard reaction but those intensity were disable to enfold the "goaty" flavor. Therefore, the integration to reactive amino acid, such as methionine is required to enhance the production intermediate product of maillard reaction resulting in the huge suppression to off-flavor in goat's milk. Methionine is a remarkable reactive compound that has ability to produce high content of carbonyl compound; an intermediate compound from maillard reaction. Therefore, this research will integrate methionine and rare sugars in maillard reaction process to produce high content of intermediate product of maillard reaction to suppress the off-flavor in goat's milk. This research will be done for 2 years and this 1st research will be focused for (1) production of strong carbonyl compound (quinoxalines) from the reaction mixture containing methionine and rare sugars (2) one patent document, (3) one research paper in Emirates Journal of Food and Agriculture (Scopus, IF= 0.3) and (4) one proceeding's article at International Conference on Renewable Energy and Development, Japan (Scopus Indexed Proceeding). To the best our knowledge, there was no research about this and this research will be provide high value result. We believe that this research also provide a strategy to produce the less or no goaty flavor in goat's milk with ease.

Keywords: carbonyl compound, quinoxalines, goaty flavor, maillard reaction, rare sugars.

CHAPTER I. INTRODUCTION

Background Study

Goat has known as tropical livestock, which is one of tropical country is Indonesia. The ability of goat to growth in tropical area like Indonesia should increase the population. Unfortunately, its population in Indonesia is still low and stagnant.

Table 1. Population of goat and cow in Indonesia from 2010–2015

Commodity	2010	2011	2012	2013	2014	2015	Yearly Growth Rate (%)
Goat (x1000,000)	15.4	15.8	14.5	15.1	15.6	16.3	1.2
Cow (x100,000)	3.6	3.6	3.7	4.5	4.8	5.1	8.3

Source: (Dirjen–Peternakan, 2016)

As seen on Table 1, the growth rate of goat's population in five years is only 1.2%, about one seventh from cow's population. These data showed that the growth of goat's population is not only still low in population, but also in non-significant improvement. This condition was effected by several factors, i.e.: (1) farmer's leak in capability of technical management or animal handling resulting in the high bacterial contamination (Al-Baarri et al., 2010, Nawangsari et al., 2014, Rasbawati et al., 2014, Villa et al., 2014, Al-Baarri et al., 2015), (2) the change of climate may also amplifies the animal disease resulting in high mortality, (3) the high number of slaughtering female livestock, resulted in the decrease of the regeneration rate (Makka, 2004, Ginting, 2006, Heriyadi, 2008).

The demand was also a prominent problem in goat's milk. There was no significant increase in the consumption of goat's milk (Dirjen–Peternakan, 2016). This fact is due to the limitation factor in goat milk: "goaty" flavor (Legowo et al., 2006a). Our research group found that consumer preference is significantly rely on the flavor in milk. Most of panelists (81%) dislike the flavor of goat milk (Al-Baarri et al., 2003). This unpleasant flavor from goat milk inhibiting its diversification product (Rachman et al., 2014). Our previous research showed that the "goaty" flavor was rely on four fatty acids: caprilic, capric, palmitic, and miristic acid (Legowo et al., 2006b, Rachman et al., 2014); (Al-Baarri and Murti, 2003).

We assumed that addition flavor–binding compound is needed to reduce the intensity of "goaty" flavor. Rare sugars has already known as the sugars from isomerase processes that produced derived sugars (mostly reducing sugars) such as psicose, fructose, and tagatose (Al-Baarri et al., 2011, Hayashi et al., 2012). This sugars plays significant role as reactive–binding component in food through maillard reaction since the production of carbonyl components could be enhanced. Therefore, our research will be conducted in the increase in the quantity of carbonyl compounds through maillard reaction process in goat milk.

Maillard reaction is chemical reaction between amino acids and reducing sugars during heating that resulted in the product of melanoidins and provide the discoloration (browning) of the mixture (Sun et al., 2006c, Zhang et al., 2009, Zielinski et al., 2010) and our research team was also work in the maillard reaction processes in food resulted in the remarkable outcome of flavor changes (Sun et al., 2006c, Kitakubo et al., 2009, Hadipernata et al., 2010a, Hadipernata et al., 2010b, Hadipernata and Hayakawa, 2012).

Rare sugars have large potential for a wide range of applications (Sun et al., 2006a). For example, D-psicose and D-allose have a potent inhibitory effect on the production of reactive oxygen species and have an ability to reduce specific aroma such as the unpleasant aroma of egg and fish in the various products like pudding, cake, noodle, sausage, and surimi (Sun et al., 2006b, Sun et al., 2007, Puangmanee et al., 2008, Hayakawa et al., 2010).

The reduction of original flavor is desired in this research, however our previous data mentioned that the maillard process was unable to remarkably reduce an off flavor in goat's milk (Rachman et al., 2014, Legowo and Al-Baarri, 2015), therefore we assumed that the elevation in responsible compound for goaty flavor reduction is advised. Thus this research will be focused in the increase in level of carbonyl group through the addition of reactive amino compound of methionine.

Our research will be arranged for (1) production of high quantity of carbonyl compound through maillard reaction between rare sugars (psicose, fructose, and tagatose) and methionine, (2) the application high carbonyl compound to goat's milk for remarkable reduction of goaty flavor. The research will provide the high value data and technology to reduce the off flavor in goat milk. Our research is original

work and have never be done previously, so international publication should be achieved with ease.

Outcome of this 1st research will be **(1) product of high content of carbonyl compound** from rare sugar and methionine integration, **(2) one patent document of high content production carbonyl compound (3) two international article publications in SOPUS**: one international journal article and one proceeding article in every year (2016 and 2017). Detail of description about international publications is presented in Table 1.

This research will produce the technology to eliminate or suppress the off-flavor in goat's milk by utilization intermediate product from maillard reaction. The benefit of this research: (1) providing the information to produce the high content of carbonyl compound through maillard reaction between methionine and rare sugars, (2) providing the strategy to suppress off-flavor in goat's milk, thus the problem of low demand in goat's milk will be solved.

CHAPTER 2. RESEARCH IN DETAIL

Our research track record

"Goaty" flavor is an exotic flavor of goat milk and bring to the sensation of goat milk consumption. It has been understand that this flavor reduced the consumer preference to consume goat milk (Al-Baarri et al., 2003, Haenlein, 2004). Our previous research was able to reduce "goaty" flavor in goat milk slightly (Al-Baarri and Murti, 2003, Legowo et al., 2006b). Within five years experiment, we made pasteurized milk, pudding, and powdered milk from goat milk and analyzed fatty acid compounds and "goaty" flavor intensity resulting in the no–significant–changes in fatty acids and no–significant–decrease in "goaty" flavor of goat milk based product (Legowo et al., 2006a). Our experiment was also continued into making cheese, yogurt, and buttermilk fat and also resulting in no–significant–decrease in fatty acid compounds and "goaty" flavor intensity (Legowo et al., 2006b, Legowo, 2011, Rachman et al., 2014).

Our result concluded that the physical treatments of goat milk did not change the fatty acid composition resulting in the non-significant-change in the intensity of "goaty" flavor. This is in line with other researcher working in the goat milk and fatty

acids (Haenlein, 2004, Seifu et al., 2004, Uysal-Pala et al., 2006, Raynal-Ljutovac et al., 2008, Silanikove et al., 2010). We believe that the enhancement compound that was responsible for "goaty" flavor reduction should be elevated (Legowo and Al-Baarri, 2015). This responsible compound is carbonyl compound that can be elevated trough the reaction between methionine and reducing sugar (Pfeifer and Kroh, 2010). As our knowledge, no researchers have been tried to produce high carbonyl content using high capacity of reducing sugar such as rare sugars and reactive amino such as methionine. Furthermore, no researcher investigated goaty flavor intensity through the elevation of carbonyl group.

Our current research focused on the production high content of carbonyl compound through maillard reaction comprising methionine and rare sugars: fructose, psicose, and tagatose in the reaction and for the next step, the high content of carbonyl compound will be applied to pasteurized goat's milk.

Our goal will be achieved in 2018 or 2019 with the production of goat's milk with very less amount of goaty flavor.

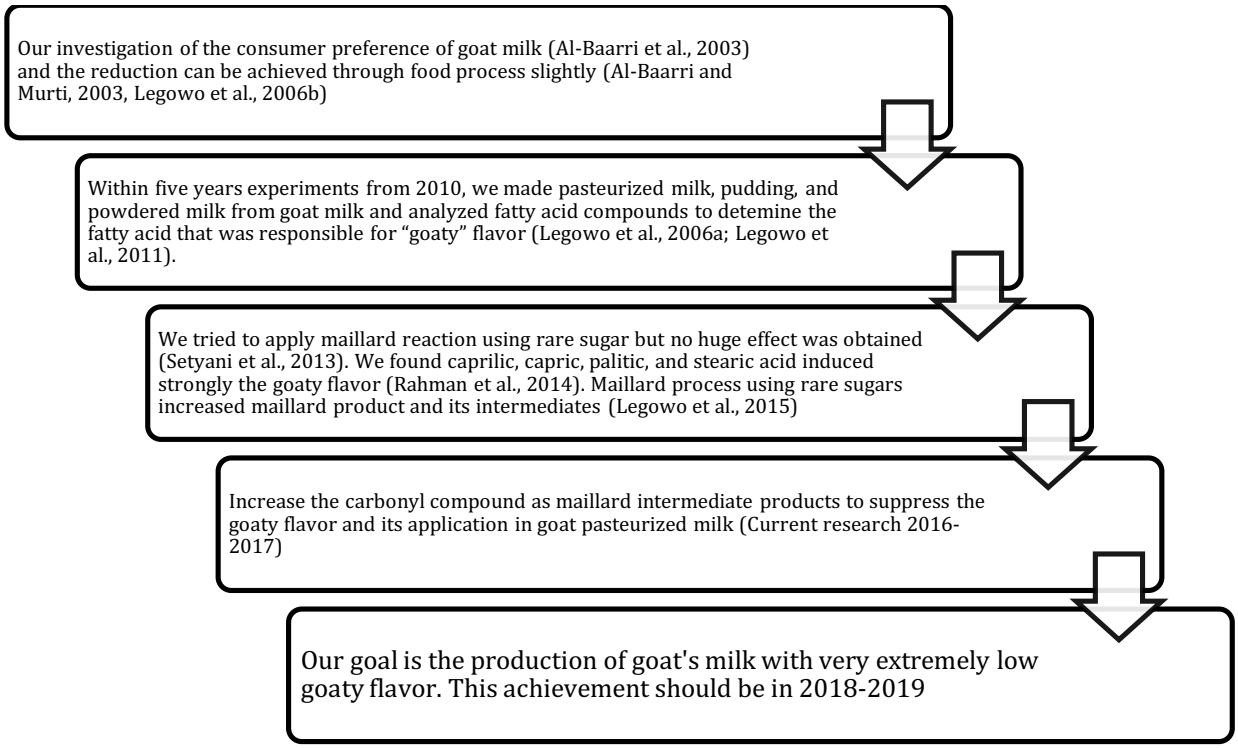

Figure 1. Track record of experiment about reduction of goaty flavor. All of experiments above were conducted by A. M. Legowo as team leader.

Outcomes of research

Four products will be generated from this research in 2016:

1. the product of high content carbonyl compound through maillard reaction between methionine and rare sugars: fructose, psicose, and tagatose
2. one patent document of "producing high content of carbonyl compound from maillard reaction between fructose/psicose/tagatose and methionine"
3. one international publication in Scopus indexed journal (Table 1)
4. one international publication in Scopus indexed proceeding (Table 1)

Table 2. Research outcomes for international publications indexed by SCOPUS

Year	Publisher	IF and indexing	Title	Authors
2016 SCOPUS-Indexed International Journal	Emirates Journal of Food and Agriculture pISSN : 2079-052X eISSN : 2079-0538 www.ejfa.me	0.31 • Thomson Reuters • SCOPUS • CAB	Investigation of methionine reaction in maillard process with rare sugar integration to produce carbonyl group	Anang M Legowo Ahmad N Al-Baarri Shigeru Hayakawa Masahiro Ogawa
2016 SCOPUS-Indexed International Proceeding	ICRED 2016 Renewable Energy and Development Proceeding will be Published at MATEC Web of Conferences (ISSN: 2261-236X) http://www.icred.org/proceedings.html	• SCOPUS • CPCI (Web of Science)	Reaction rate of fructose as reducing sugar in methionine-fortified maillard process	Anang M Legowo Ahmad N Al-Baarri Shigeru Hayakawa Masahiro Ogawa
2017	Two destinations: Emirates Journal of Food and Agriculture and Scopus Indexed Proceeding in 2017	SCOPUS	Topics: Methionine integration for carbonyl production to reduce goaty flavor in pasteurized milk	Anang M Legowo Ahmad N Al-Baarri Shigeru Hayakawa Masahiro Ogawa

Four products will also be generated from this research in 2017

1. the product of goat milk with no or very less amount of goaty flavor through the application of high carbonyl compound into goat milk (pasteurized milk)

2. one patent document of "involvement carbonyl compound from maillard reaction to produce very less amount of goaty flavor in goat's milk"
3. two international publications: in Emirates Journal of Food and Agriculture (SCOPUS) and international proceeding in 2017.

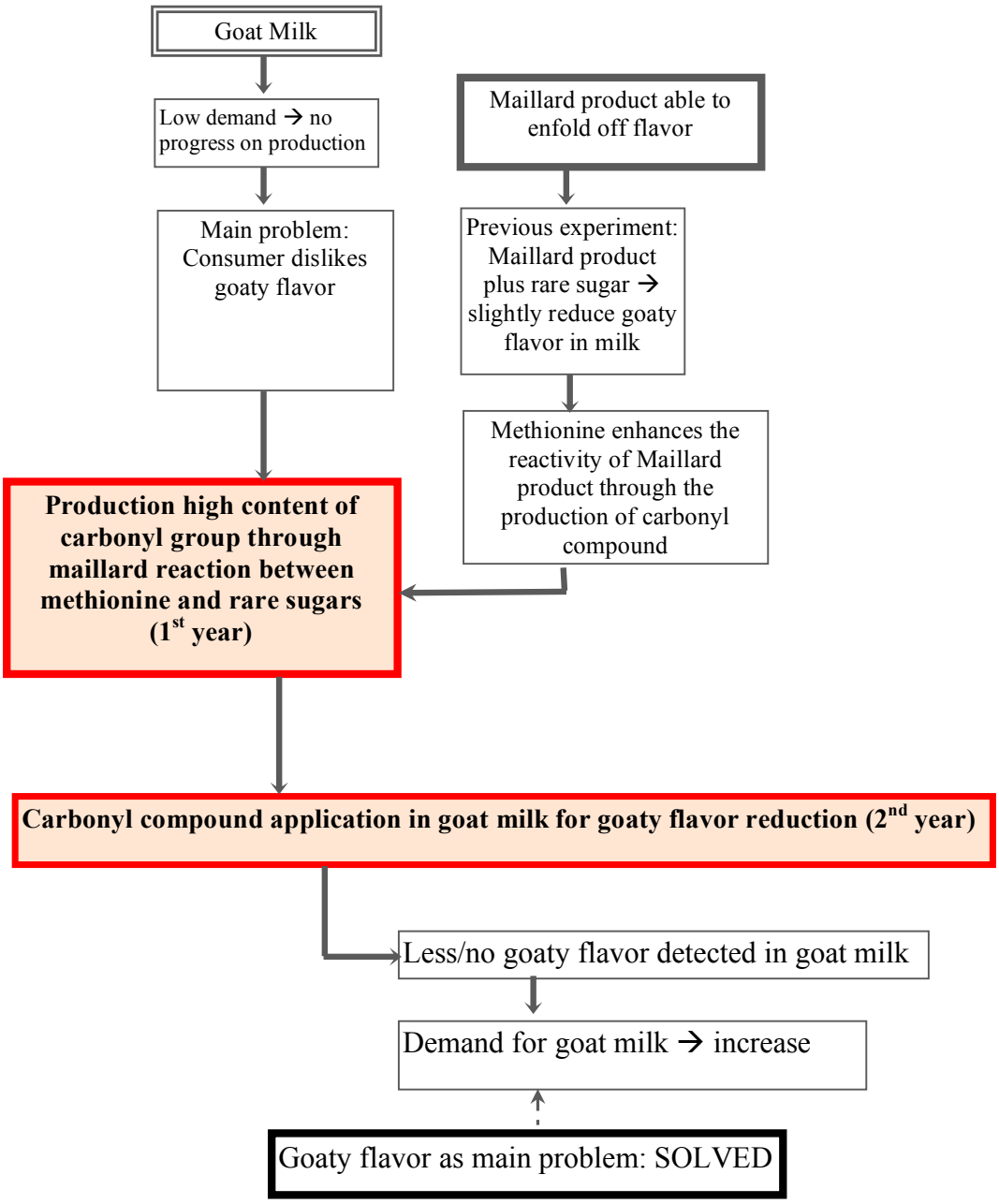

Figure 2. Flow chart of general research

The Urgency of Research

The growth rate of goat population is extremely low (about 1% per year) as compare to the growth rate of cow (about 8% per year). One of the reasons of this condition is the less demand for goat's milk. Consumer, including the milk industry, did not prefer using goat milk due to unpleasant "goaty" flavor. The reduction of this unpleasant flavor seems the only way to increase the consumer's favor resulting in the increase in its demand. Finally, high demand may promote the increase the population, then the problem of low in population of goat can be solved.

Up to now, there is no research successfully done to reduce the "goaty" flavor of goat milk including our previous research. We conducted serious experiments in order to reduce or enfold "goaty" flavor, however, still unable to find the solution to reduce the intensity of "goaty" flavor. Our experiment was then on maillard process to reduce goaty flavor. It has been known that methionine were able to produce carbonyl compound, a product from maillard reaction that are responsible in reducing or enfolding aroma. This good news provide the idea to our team to utilize rare sugars that has been known as reactive component, in the maillard reaction to produce high content of carbonyl compound.

No researches have been reported about the involvement of rare sugar and methionine to produce carbonyl compounds, therefore this research is urgently needed to solve the problem on the reduction of goat flavor. We believe that this research will result the beneficial strategy to increase a demand of goat milk through the reduction of its goaty flavor and publishable in international journal.

CHAPTER 3. RESEARCH METHODS

Principal work for production carbonyl compound

Maillard reaction will generate the final product and intermediate product of maillard reaction product. Carbonyl compound is the intermediate product of maillard reaction, therefore the mixture reaction containing sugar and amino acid will be stopped using 1,2-diaminobenzene. The temperature of 60–130˚C will be applied to start the maillard reaction. The mechanism of intermediate production of maillard reaction is presented in Figure 3.

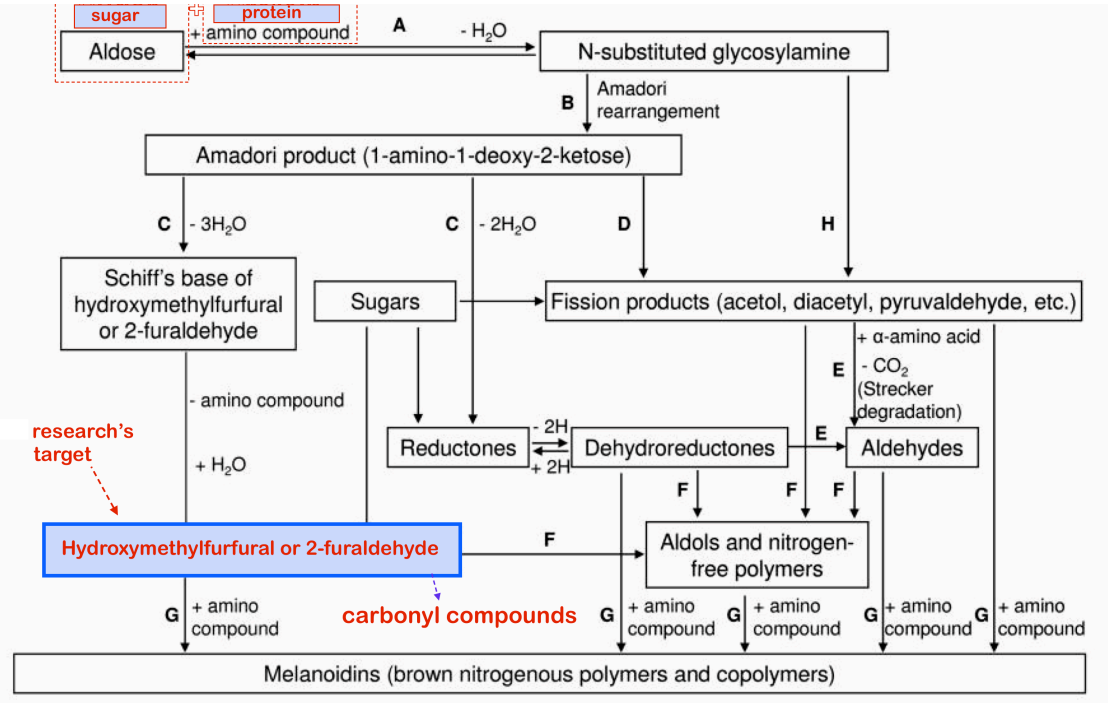

Figure 3. Carbonyl generation during the maillard reaction

Procedure for carbonyl compound generation

Production of carbonyl compound will be done as previously mentioned method by Pfeifer and Kroh (2010) with slight modification. The non-enzymatic reaction will be prepared to compose an aqueous solution of 1 mM rare sugar and 1 mM amino acid. L-methionine will be selected as component to produce carbonyl since the L form provide strong reaction to sugar components. As much as 10 mg L-methionine will be diluted in 10 mL of H_2O containing 10% fructose, psicose, and tagatose and then the reaction solution will be adjusted to a pH of 3.0–9.0 with 3.0 N HCl or 3 N NaOH.

This model solutions will be heated in sealed ampules at 100–130°C for up to 300 min in a thermo block. After a defined reaction time, 500µL of the samples will be trapped with 500µL of 0.05 M 1,2-diaminobenzene solution to intercept the R-dicarbonyls as quinoxalines. After 3 h at 25°C, quinoxalines will be analyzed by HPLC and GC-MS after acetylation. SDS PAGE will be used to determine the new compound production from maillard reaction.

High-Performance Liquid Chromatography (HPLC)

HPLC will be used for separation the quinoxalines from reaction model. This research will be fully done in Integrated Laboratory, Diponegoro University using HPLC Shimadzu LC-10 AT. The thermostat will be set at 30°C, Shimadzu CT0-6A will be used as guard column and Nucleosil C18 Macherey-Nagel will be used as main column. Sample injection will be 40µL. The eluent is methanol/water gradient with flow rate 1 mL/min. This method is adapted from the previous research (Pfeifer and Kroh, 2010).

Gas Chromatography Mass Spectrophotometry (GC-MS)

The samples will be extracted with n-butanol. The solvent will be dried off, the residue will dissolved in toluene, and acetic anhydride was added. Specification for gas chromatography instrumentation: capillary column is BPX 5 (SGE, 30 m, 0.25 mm i.d.,0.5µm film thickness); carrier gas is helium 4.6; injection temperature is 270°C; with split 1:10; initial temperature will be set at 95°C, held for 1 min, raised from 95 to 200°C at 15°C/min, 200°C held for 1 min, raised from 200 to 280°C at 3°C/min, 280°C held for 5min, raised from 280 to 300°C at 5°C/min, 300°C held for 5 min. Column effluents will be analyzed by selected ion monitoring (SIM). Quantification was carried out by comparison of peak areas. This procedure is adopted from other researcher (Pfeifer and Kroh, 2010)

SDS PAGE for determining maillard reaction product

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), will be used for determination the maillard reaction product. The new compound of maillard reaction product will be determine easily through the formation new band in the upper side of SDS gel. This application will be done as a procedure by Al-Baarri et al. (2011).

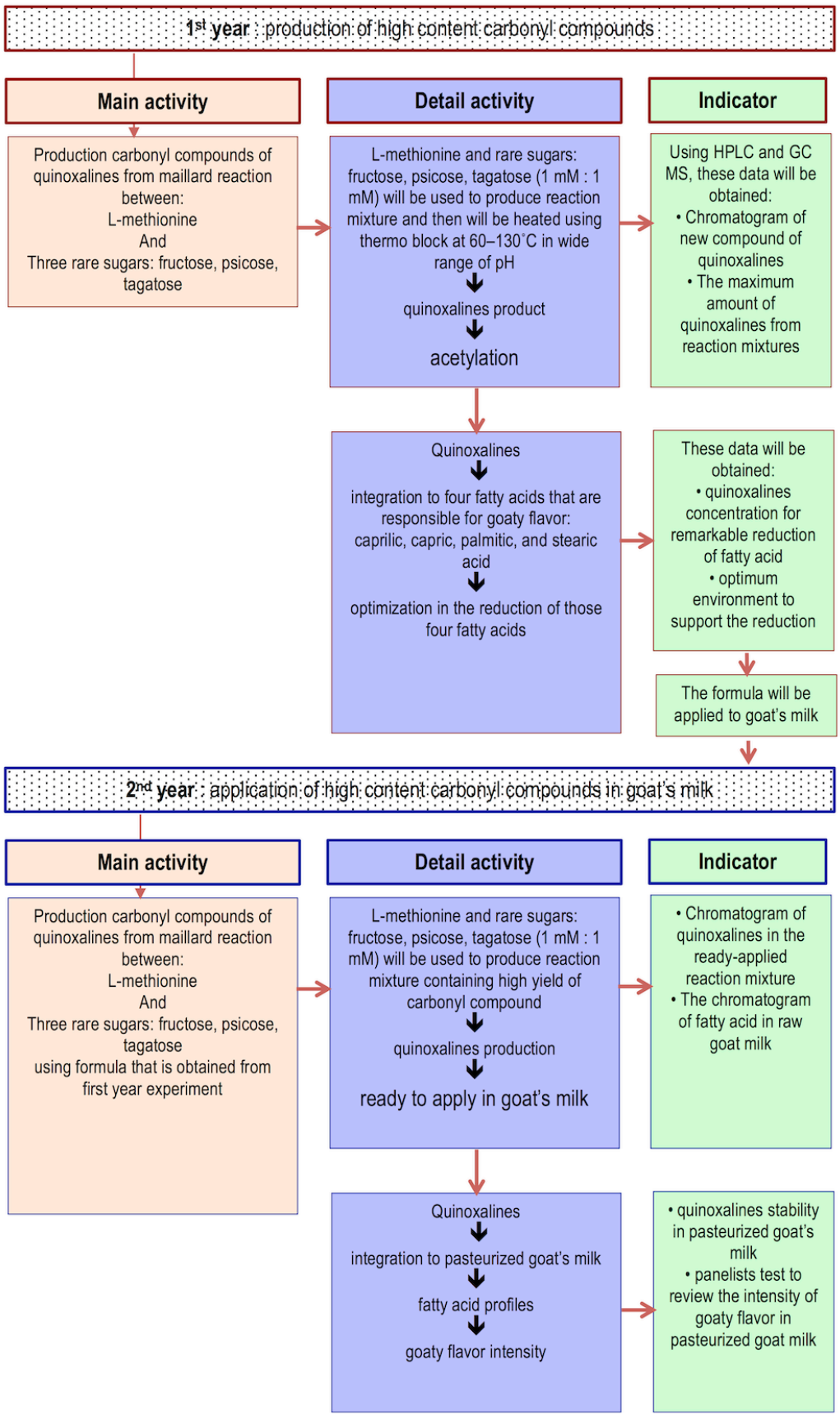

Figure 4. Flow chart of whole research method in two years

CHAPTER 4. RESULT AND DISCUSSION

Antioxidant is the most popular nutrient compound, that should be exist in food and food product. It is now being developed by manufacture of food product and to be a final target to increase the quality of product manufacture (Madhavi *et al.*, 1995). Antioxidant is key rule for maintaining the human health especially for the person who need a demand status for health example elder people (Sarojnalini and Sarjubala, 2014). Since various food spread into various menu that produce negative effect example acrylamide or radical compound that maybe produce from food, so antioxidant is really to be needed (Peralta, 2010). The antioxidant can be obtained from fruits and vitamin, but antioxidant from natural compound is still rare. Various chemical antioxidant is commonly used for examples are BHT and BHA. Maillard reaction is reaction based betwen sugar and protein producing Hydoxymethylfurfural (HMF) (Packer, 1996). This compound still unknown but intermediate compound can produce high antioxidant activity, therefore the maillard reaction should be produce high antioxidant compound. In this research we try to utilized by product of milk and sugar to produce high oxidative compound for various puposes.

Maillard reaction between whey and D-psicose is a non enzymatic browning reactions that occur because of the reaction of amino acids from whey and reducing sugar of D-psicose. D-psicose is a rare sugar that is reactive to the formation of glycation reactions that produce the brown color and produce antioxidant compounds that make up the flavor (Tang, 2012). Maillard reaction contributes to the formation of brown pigment on the food, but also it can produce antioxidants (Panglossi, 2006). The existence of a complex constituent in whey and D-psicose is expected to produce glycation reaction with the complete furfural compound, because furfural compound contributes to the formation of color and flavor for the Maillard reaction (Wong *et al.,* 2008). The purpose of this research is to find the optimum time of heating to get the maximum browning intensity and antioxidant activity.

Table1. Absorbance at 420 nm while heating for 5 until 40 hours with 55^{o}C heat on Maillard Reaction of whey and 0,5% D-psicose.

Time of Long Heating (hours)	Absorbance (Abs)
5	0,552
10	0,731
15	0,641
20	0,659
25	0,682
30	0,826
35	0,689
40	0,789

Based on the Table 1, it can be concluded that long heating from 5 hours untill 40 hours showed the increase of Browning Intensity which indicated from absorbance value. At the long heating 5th resulted absorbance 0,552, 10th resulted absorbance 0,731; 15th resulted absorbance 0,641; 20th resulted absorbance 0,659; 25th resulted absorbance 0,682; 30 resulted absorbance 0,826; at 35th and 40th resulted in the decrease of absorbance are 0,682 and 0,789. Based on the data can be concluded that the long heating treatment given the high browning intensity.

Maillard reaction is a complex reaction consisting of five complex stage are amine carbonyl condensation, amadori rearrangement, the formation of pigment, strecker aldehyde degradation, and polymerization. The formation of brown color was formed at the amadori rearrangement and pigment formation phase (Sun *et al.,* 2006). The longer time of heating causes the water on whey and 0,5% D-picose evaporate then OH chain was eliminated and forming schiff base. Schiff base will transformed to be HMF. HMF will accumulated and formed the melanoidin (Bastos *et al.,* 2012*)*. Melanoidin known as forming the brown color on Maillard reaction. The longer heating time on maillard reaction, the melanoidin that forms and causes brown color also increasing (Schebor *et al,* 2014*.)*. Color is one of indicators for the occurrence of a change chemical due to glication in food products (Flogiano, 2014).

Table 2. Color development of maillar reaction product during storage

Time of Long Heating (hours)	Absorbance (Abs)
5	1,190
10	1,587
15	2,346
20	7,656
25	27,303
30	17,945
35	2,682
40	1,675

Based on table 2, it can be concluded that long heating from 5 hours untill 40 hours showed the increase of antioxidant activity which indicated from absorbance value. At the long heating 5th resulted absorbance 1,190, 10th resulted absorbance 1,587; 15th resulted absorbance 2,346; 20th resulted absorbance 7,656; 25th resulted absorbance 27,303; at 30th, 35th and 40th resulted in the decrease of absorbance are 17,945; 2,682 and 1,675. Based on the data can be concluded that the optimum time of heating at 25th hours with the absorbance 27,303.

The increase of antioxidant activity allegedly is the result of the reaction between a amine of protein on whey and carbonil compound on sugar d-psicose that produces antioxidant compounds (Izumori *et al.,* 2005). Antioxidant formed at some stage of the reaction, and for maillard reaction the optimum time was at 25th hours that produces intermediete compound with the assumption that maillard reaction had reach out the final stage (Zamora and Hidalgo, 2005). From the data obtained, it is known that the longer time of heating would increase the antioxidant compound at some stage and reached maximum ammount in 25th hours.

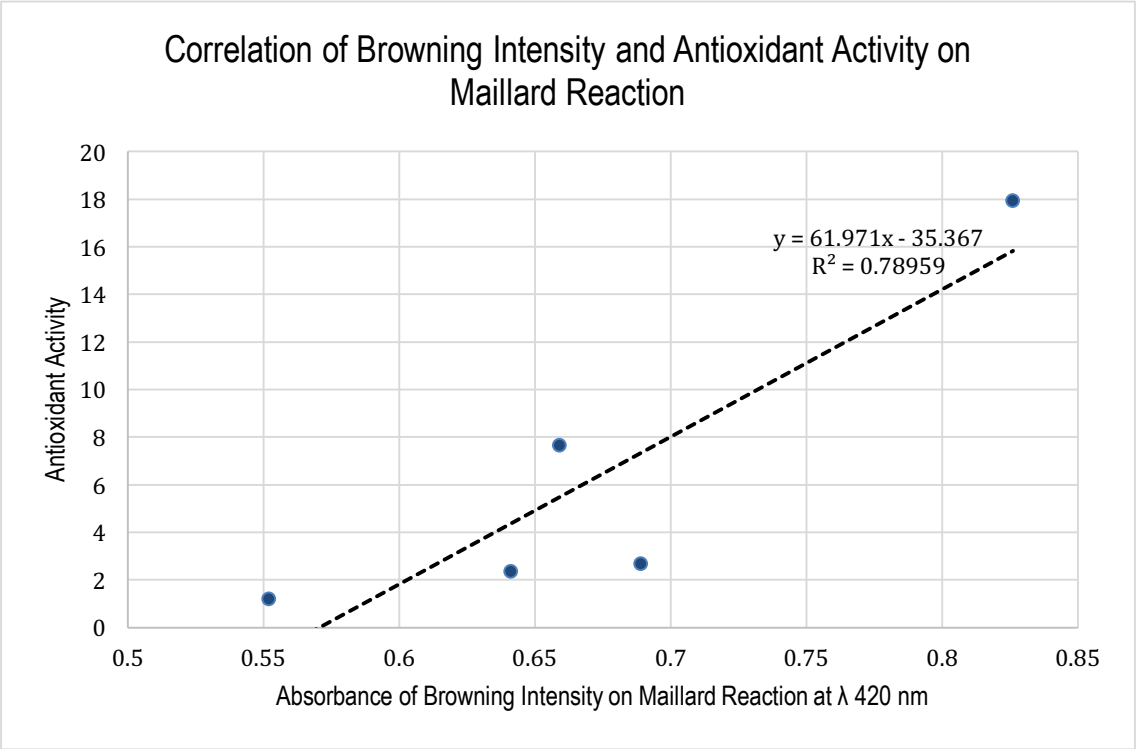

Figure 1. Correlation of Browning Intensity and Antioxidant Activity on Maillard Reaction.

Browning intensity of Maillard reaction in whey and rare sugar D-psicose also had positive correlation with antioxidant activity following the equation : y = 61,971x – 35,367, with R^2 = 0,7896 (Figure 1). These equations indicated that the browning intensity had close correlation with antioxidant activity. The close correlation of browning intensity and antioxidant activity was indicated by the determenation value (R^2) of the equations reached 78%. This means that solution of whey and 0,5% D-psicose measured at λ 734 nm contributed to antioxidant activity.

Maillard reaction product (MRP) is natural antioxidant formed during Maillard reaction (Phisut and Jiraporn, 2013). This antioxidant formed as a result of whey and 0.5 % D-Psicose through several levels Carbonil-amine heating, and also the process of degradation amadori compound to be amino reduction that produces antioxidant activity. The more long time of heating will increase the browning intensity, the brown color formed from intermediet compound (Melaonidin) (Sun *et al., 2006)*. Melanoidin is known have high antioxidant value. D-psicose is important thing to establish the glication on Maillard reaction that produces antioxidant compounds (Martins *et al.*, 2001).

CHAPTER 5. CONCLUSION

D-psicose generated rapid browning intensity on maillard reaction product in low temperature of heating and less storage of drying process. This research may provide the benefit for the application of D-psicose to produce food product based on maillard with low heating and short process.

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PHOTO FROM OUR RESEARCH

Picture 1. Raw milk

Picture 2. Whey preparations

Picture 3. Filtering Whey

Picture 4. Ovening sample

Picture 6. Microplate reader for analysis

Picture 7. Result for 5th hours

Picture 8. Result for 10th hours

Picture 9. Result for 15th hours

Picture 10. Result for 20th hours

Picture 11. Result for 25th hours

Picture 12. Result for 30rd hours

Picture 13. Result for 35th hours