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Chemical and biochemical characterization of soybean produced under drought stress

Caracterização química e bioquímica de soja produzida sob condições de déficit hídrico

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Abstract

Brazil is the second soybean (*Glycine max* L. Merrill) producer and exporter in the world. In 2005, soybean cultivated in the southeastern region of the country suffered drought stress imposed by adverse high temperatures and low humidity during its reproductive stage. Little information is available regarding the effect of drought stress on the quality of grains. In this study chemical and biochemical characteristics of five soybean samples belonging to three different cultivars grown under drought stress were evaluated. The samples did not meet standards for marketing and contained high amounts of green seeds. Grains were analyzed for appearance, 100 seed weight, humidity, water activity, proteins, lipids, lipoxygenase *1* activity, peroxides, and pigment contents after harvest and after 20 months of storage at room temperature. Acidity was measured also after 30 months of storage. The values of water activity and humidity were 0.6-0.7 and 8.7-11.9%, respectively, and they did not change during storage time, but there was an increase in acidity, which alludes to lipase activity. The activity of lipoxygenase 1 was greatly affected. Immediately after harvest, the green pigments were represented mainly by pheophytin *a*, followed by pheophytin *b*, small quantities of chlorophyll *b* and chlorophyll *a,* and traces of other chlorophyll derivatives. After 20 months of storage almost all green pigments had disappeared. Drought stress probably enhanced membrane permeability, which led to a lower pH and promoted transformation of chlorophylls to pheophytins.

Keywords: *Glycine max; quality of grains; climate conditions; storage.*

Resumo

O Brasil é o segundo maior produtor e exportador de soja (*Glycine max* L. Merrill) do mundo. Em 2005, a soja produzida no sudeste do Brasil sofreu estresse hídrico devido a temperaturas altas e umidade baixa durante o período reprodutivo. Pouco se sabe do efeito de déficit hídrico sobre a qualidade dos grãos. Neste trabalho, foram analisadas características químicas e bioquímicas de cinco amostras de soja, pertencentes a três cultivares que haviam sofrido déficit hídrico durante a fase de maturação. As amostras não atenderam às especificações para comercialização e continham elevado teor de grãos verdes. Foram analisados aparência, peso de 100 grãos, umidade, atividade de água, proteínas, lipídios, atividade de lipoxigenase *1*, acidez, peróxidos e presença de pigmentos após a colheita e após 20 meses de armazenamento em temperatura ambiente. A acidez foi medida adicionalmente após 30 meses de armazenamento. A atividade de água e a umidade mantiveram-se constantes durante todo o período e variaram entre 0,6-0,7 e 8,7-11,9%, respectivamente, mas houve aumento de acidez, o que implica em atividade de lipases. A atividade da lipoxigenase foi fortemente prejudicada. Imediatamente após a colheita os pigmentos predominantes foram feofitina *a* e *b*, quantidades reduzidas de clorofila *b* e *a* e traços de outros compostos de degradação da clorofila. Após 20 meses de armazenamento, quase todos os pigmentos verdes haviam desaparecido. O estresse hídrico provavelmente aumentou a permeabilidade de membranas o que provocou redução de pH e promoveu a transformação de clorofilas em feofitinas.

Palavras-chave: *Glycine max; qualidade dos grãos; condições climáticas; armazenamento.*

1 Introduction

Soybean is one of the most important agricultural products worldwide, Brazil is the second largest producer after the USA (BRAZIL; CONAB, 2007). In 2006, soybean contributed with a production of 58.4 million tons to the Brazilian exports with about US$ 9.3 billion, which represented about 6.8% of the total export (BRAZIL; EMBRAPA, 2007).

The greatest decline of commercial soybean production in the southeast of Brazil in the history of Brazilian agriculture occurred in 2004 and 2005. It was caused by abnormal climatic conditions with high temperatures and a lack of adequate rainfall, which affected the development of the seeds, led to impaired quality, and reduced its commercial value. Apart from the reduction in yield, there was a significant amount of seeds that maintained their green color even after harvest and drying (BRAZIL; CONAB, 2007). Post harvest drying is required to reduce moisture to a maximum of 14%, which is the upper limit considered for safe storage.

The Brazilian legislation allows 8% of greenish seeds for marketing (BRAZIL; MINISTRY OF AGRICULTURE, 2007) while the legislation in the USA is more demanding for

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international trade standards and established a maximum level of 1% of greenish seeds for top grade soybeans (MANGOS; BERGER, 1997).

The green color of the seeds is a result of the presence of a high amount of chlorophylls and their derivates. Most of them are fat-soluble compounds and therefore are extracted with the oil resulting in a darker coloration. Since the shelf life of the oil and the efficiency of the catalyzer during eventual hydrogenation processes are reduced in the presence of chlorophylls, additional bleaching steps are required to remove these pigments raising significantly the production costs (TAUTORUS; LOW, 1993).

Under normal harvest and drying conditions on the field, chlorophyll is naturally completely degraded to colorless and more polar derivatives due to the opening of the tetrapyrrole macrocycle (HEATON; MARANGONI, 1996). This catabolism can be understood as a detoxifying process for the plant due to the loss of photodynamic and pro-oxidant properties of this pigment (HÖRTENSTEINER, 2004; PRUŽINSKÁ et al., 2003). However, environmental factors during the ripening stages which lead to a rapid decrease in water content impair this natural degreening. In general, the degreening is blocked by high temperatures and little rainfall, as well as by premature crop followed by fast drying processes at temperatures above 40 °C (GOMES et al., 2003).

Abnormal weather has played a huge role in the sharp decline in soybean yields and efforts have already been made to understand the influence of growing conditions and drying processes on the retention of green pigments in the seeds. So far, no study has been reported on the physicochemical characteristics of soybean that had suffered drought stress. Therefore, the present work was carried out in order to measure the effect of drought stress on several quality-related parameters before and after storage.

2 Materials and methods

Soybean samples. Five samples of commercial Brazilian soybean (*Glycine max* L. Merrill) were analyzed including the flowing varieties: Embrapa BRS 184 (two), Embrapa BRS 133 (two), and Coodetec 201(one). All samples were produced by farmers in the region of Palmital in the state of São Paulo in 2004/2005 and graded by the São Paulo General Warehousing and Centers Company (CEAGESP). The samples were harvested at the stage of full maturity (R8) between 126 and 132 days after plantation following the scale reported by Fehr and Caviness (1977). Considering the quality standards for marketing, none of these samples met the Brazilian standards due to the presence of mainly green-colored, damaged and broken seeds, and impurities. At CEAGESP, pods were removed and seeds were dried under a hot air stream at 100 °C until reaching moisture content less than 14%, value determined by law. The humidity had to be reduced to avoid the development of microorganisms and mycotoxins and diminish seed fermentation and auto degenerative biochemical reactions. The internal temperature of the seeds reached approximately 60-70 °C during this procedure. Later, the seeds were transported to our laboratory, where they were stored in the dark at room temperature in sealed plastic boxes for up to 30 months. In order to conduct analyses, soybean seeds were selected excluding impurities and split and broken kernels were ground in a laboratory mill (Kinematica A10 Analysenmühle, Switzerland). All following analyses were made in triplicates, unless differently mentioned. *Moisture content*. Five grams of crushed soybeans were dried in an oven at 105 °C until constant weight and moisture contents were determined by the difference in weight before and after drying (INSTITUTO ADOLFO LUTZ, 1985). *Water activity*. Approximately two grams of ground soybean were analyzed at 25 °C using a Novasina Thermoconstanter Humidat (Switzerland) according to the methodology described by Johnston and Lin (1987). *Analysis of lipids*. Total lipids were determined by continuous extraction of five grams of soybean flour during eight hours with 300 mL diethyl ether in a Soxhlet extractor (INSTITUTO ADOLFO LUTZ, 1985). The proportion of lipids in the sample was based on dry-mass. *Protein content*. Soybean flour was defatted and 30 mg were used to analyze the N content by micro-Kjeldahl (N × 6.25). *Oil extraction*. Approximately 50 g of soy flour was soaked with n-hexane for 18 hours at room temperature. The solvent was eliminated under vacuum (INSTITUTO ADOLFO LUTZ, 1976). *Acidity*. The extracted oil was analyzed for the presence of free fatty acids after the dissolution of two grams of oil in 25 mL of alcohol/diethyl ether (2/1; v/v) and titulation with 0.1 M potassium hydroxide using phenolphthalein as indicator (INSTITUTO ADOLFO LUTZ, 2005a). *Presence of peroxides*. The extracted oil was analyzed for peroxides after the dissolution of five grams of oil in 30 mL of chloroform/glacial acetic acid (3/2; v/v), 0.5 mL of saturated potassium iodide and 30 mL of water and subsequent titulation with 0.01 N sodium thiosulfate (INSTITUTO ADOLFO LUTZ, 2005b). *Hundred-seed weight*. Each variety was measured in triplicate excluding broken and injured seeds. *Lipoxygenase 1 activity*. According to the methods of Axelrod, Cheesbrought and Laasko (1981) and Oliveira et al. (1998), water-soluble proteins were extracted from 20 mg of soybean with 1.2 mL extraction buffer (60 mM Tris, 15 mM $CaCl_2$ and 13% sacarose; pH 8.2). The suspension was centrifuged at 14000 × g at 4 °C for 20 minutes and the supernatant was used for spectrophotometric analysis of lipoxygenase activity. Briefly, 2,5 µL of the supernatant in 1.0 mL 0.1 M sodium borate buffer (pH 9.5) was added to 6.0 µL of 10 mM sodium linoleate as substrate (0.31% linoleic acid, 0.36% Tween-20, 2M NaOH) and the formation of hydroperoxides of linoleic acid was monitored by measuring absorbance at 234 nm during two minutes. The enzymatic activity was expressed as units of enzyme activity/mg soybean flour (dry weight). *Spectrophotometric quantification of total chlorophylls.* According to the method of Porra et al. (1989), 8.0 g of soybean flour was suspended in 10 mL of 80% acetone and samples were centrifuged at 15000 × g for 15 minutes. The supernatants were analyzed spectrophotometrically at 663.6 e 646.3 nm (Shimadzu, Tokyo, Japan UV-1650PC). The quantities of total chlorophyll were calculated using the equations proposed by Porra et al. (1989) and expressed in $\mu g.mL^{-1}$. *Analysis of chlorophylls by HPLC*. According to Sinnecker et al. (2005), five grams of soybean flour was homogenized with 30 mL of 80% acetone, filtered, and the process was repeated at least three times until all green color disappeared. Filtrates containing chlorophylls and derivatives

were combined and transferred to 100 mL petroleum ether, washed with water, and dried with anhydrous sodium sulfate. The obtained solution was dried under vacuum and dissolved in five milliliters of acetone. The pigments were separated by HPLC (Shimadzu, Tokyo, Japan, CLASS-M10A, UV/Vis) and identified and quantified by comparison with standards (MANGOS; BERGER, 1997). *Extraction and separation of colorless chlorophyll derivatives*. Exactly 0.5 g of soybean flour was homogenized with 1.0 mL 100 mM methanol/potassium phosphate buffer, pH 7, in quadruplicates and centrifuged at 13000 × g/10 min. The supernatants were transferred to clean tubes and centrifuged again. The supernatants of each sample were then combined and dried under vacuum. The pellets were dissolved in one mL of H_2O and the suspensions analyzed by HPLC (Shimadzu, Tokyo, Japan CLASS-M10A, UV/Vis) on a Shim-pack column (VP-ODS, 5 µm, 250 × 4.6 mm) with a Thermoquest pre-column (11 × 4.6 mm) using a 0.5 mL/min flow. The absorbance was monitored at 459, 320 and 210 nm and the spectrum was recorded between 190 and 800 nm with a diode array detector UV/Vis (SPD-M10AVP). The injection volume was 50 µL and the solvent system consisted of a) water; b) 100 mM K_3PO_4 buffer pH 7.0; and c) methanol. The gradient was developed as follows: 0 minute (0:80:20; v/v/v), 10 minutes (0:80:20), 70 minutes (0:40:60), 80 minutes (0:40:60), 82 minutes (20:20:60), 87 minutes (20:10:70), 90 minutes (15:5:80), 96 minutes (9:1:90), 97 minutes (4.5:0.5:95), 98 minutes (5:0:95), 100 minutes (0:0:100), 115 minutes (0:0:100), 120 minutes (80:0:20), 125 minutes (80:0:20), and 135 minutes (0:80:20) (OBERHUBER et al., 2001). *Statistical analyses*. The data obtained are presented as mean value ± standard deviation of the analyses in triplicate, unless differently mentioned. The data differences were submitted to a Tukey-test with a confidence interval of 0.95 (STATGRAPHICS, Vers. 2.6).

3 Results and discussion

3.1 Grain quality

The examination of the soybeans grown under adverse climate conditions revealed striking differences from those grown under normal conditions.

None of the samples analyzed met the quality standard for marketing imposed by law. Official grading of all samples revealed more than 8% green colored seeds and a high percentage of impurities. It must be observed that the Brazilian quality standards for soybean were altered recently in order to get closer to the more rigid international standards (BRAZIL; MINISTRY OF AGRICULTURE, 2007). According to the new standards, grains are separated into two groups: group I is destined to in natura consumption and therefore has less rigid limits; group II is meant for other uses, like oil extraction. In group II, crops can not contain more than 8% of greenish seeds, 30% of parted, broken or crumpled seeds, and 1% of impurities.

Furthermore, it was found that the seeds grown under adverse climate conditions had a lower hundred-seed weight (14.2 ± 2.2 g/100 seeds) compared with soybean samples grown under normal climate conditions (17.5 ± 0.2 g/100 seeds). In general, when soybean plants suffer drought stress, their seeds are reduced in size and weight (NOGUEIRA; NAGAI, 1988). However, quality standards do not mention restrictions related to grain size, which can be related to cultural traits.

3.2 Presence of green pigments

Spectrophotometric analyses of total chlorophyll contents conducted with the five samples revealed variable amounts with mean values of 4.88 ± 1.48 mg/kg. It was observed that the green pigments were mostly located just below the hull giving the grains a green appearance. The heterogeneity of chlorophyll distribution resulted in a lower than expected concentration of this pigment considering the greenish appearance and the amount of greenish seeds varied between 5 and 22%. Chlorophylls are naturally completely degraded during the last stages of the ripening process, and the expected final total chlorophyll levels in mature grains are lower than 1 ppm.

Therefore, an important quality property of grains is their maturity status. Consequently, the presence of green seeds is usually considered an indicator of seed immaturity, but it can also be the result of improper post harvest drying conditions or when desiccants are applied to reduce seed moisture. Elevated amounts of chlorophylls affect the seed-crushing industry since these pigments act as photo sensitizers and reduce oxidative stability and shelf-life of oils (TAUTORUS; LOW, 1993).

Analyses by HPLC revealed that immediately after harvest pigments in the green seeds were represented not predominantly by chlorophylls, but mainly by their derivatives, pheophytin *a*, followed by pheophytin *b*, small quantities of chlorophyll *b,* and chlorophyll *a* and trace amounts of other chlorophyll derivatives like chlorophyllides and pheophorbides. The abnormally high amounts of pheophytins found are probably due to an acidic environment that caused the removal of the central magnesium atom resulting in pheophytins and not caused by the enzyme Mg-dechelatase. This piece of information seems to be a clue that drought stress affected the development of the seeds during their maturation and caused membrane disorganization, which by itself enhanced permeability and a kind of cascade reaction resulting in a lower pH and promoting pheophytinization (SCHWARTZ; LORENZO, 1991). The unusual spectrum of pigments with the predominance of pheophytins has also been reported in the literature and represents soybeans harvested at different maturation stages and then dried at 40 °C or higher temperatures. Chlorophylls were retained and a high amount of pheophytins was found, ensuring the conclusion that a chemical pheophytinization was present and that these pigments were no further degraded to colorless metabolites (SINNECKER et al., 2005).

However, the green pigments almost disappeared during the 20 months of storage. After this period, only trace amounts of chlorophylls were found and the seeds turned yellow, close to those grown under normal conditions. This result suggests that the enzymatic activity is not completely blocked allowing the transformation of chlorophyll *b* into chlorophyll *a* by chlorophyll *b* reductase (ROCA et al., 2004) and subsequent degradation to chlorophyllide *a* and pheophorbide *a* by chlorophyllase and Mg-dequelatase, respectively. Afterwards, the pheophorbide *a*

monooxygenase produced "red chlorophyll catabolites" (RCC) followed by the RCC reductase forming "fluorescent chlorophyll catabolites" (FCC) (HÖRTENSTEINER et al., 1998).

It is worth pointing out that the amount of lutein, the predominant carotenoid in soybeans, did not decrease significantly during the storage time (data not shown).

Figure 1 shows the rain volume and temperatures in the region of Palmital, São Paulo, during the whole period of soybean ripening. A noteworthy lack of rainfall during the whole month of February and in the beginning of March is evident. In this late stage of soybean maturation, the degradation of chlorophylls is normally at its most active period. In this special situation the deficient rainfall accompanied by hot weather and a fast loss of grain moisture support the hypothesis to have caused partial reduction of the activity of enzymes which impaired the natural catabolism of chlorophylls. The green pigments seem to have suffered chemical pheophytizination. It is worth to remember that each enzyme has its own characteristic sensibility towards climate conditions and drought stress; therefore, their activity is directly influenced by these factors.

3.3 Enzymatic activity

The lipoxygenase *1* activity was evaluated as the indicator of endogenous enzyme activity of the grains and measured after cropping and drying by heat and after 20 month of storage. The activity varied among the samples, but they were all in the range from 7.9 to 14.8 U.mg^{-1} dry weights after cropping and postharvest drying at 100 °C (data not shown). These numbers are lower than those of the enzymatic activity found in the literature which reported values of approximately 20 U.mg^{-1} dry weights (RICE et al., 1981). However, these low enzymatic activities might be related with the post harvest heating process of the grains and not with drought stress. A reduction of lipoxygenase *1* activity by heat treatment has already been reported in the literature (RICE et al., 1981).

3.4 Acidity and chemical composition

The results from the titratable acidity are shown in Table 1. Samples were analyzed soon after cropping as well as after 20 and 30 months of storage at room temperature. The acidity increased significantly after storage, which may implicate the hydrolysis of triacylglicerols by lipases.

Nevertheless, peroxides were not found in any sample, neither after harvest nor after storage, which indicates that the antioxidant activity conferred by carotenoids like the formerly mentioned lutein present in the samples asserted oxidative stability for at least 30 months of storage.

The chemical composition of the seeds was determined to identify eventual grain immaturity at harvest time. However, the results showed that lipid and protein contents across the five samples ranged between 20.0 to 25.2 and 35.5 to 37.7 g.100 g^{-1}, respectively, and were within or slightly below the

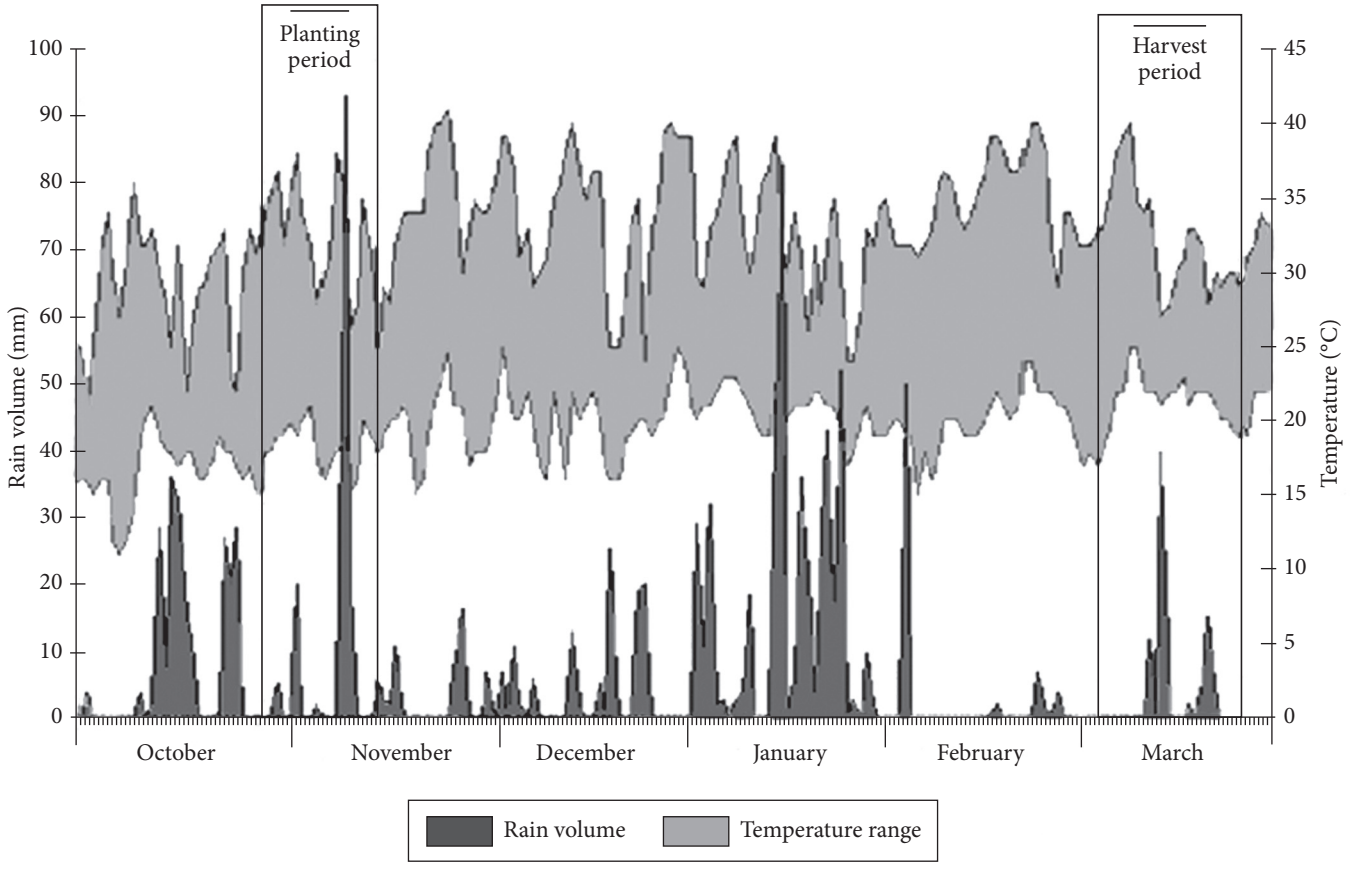

Figure 1. Rain volume and temperatures in the region of Palmital from October 2004 to March 2005.

array of amounts usually found in soybean cultivars grown under normal climate conditions. Food composition and nutrition tables (SOUCI; FACHMANN; KRAUT, 2007) report mean values of 22.1% for lipids and up to 40.5% for proteins. Apparently, drought stress did not interfere in the lipid and protein synthesis, which implies that seeds had already achieved physiological maturity. Therefore, the retention of green pigments in the seeds is not due to grain immaturity, but it seems to be caused by drought stress that had occurred at that very year.

Moisture contents were measured before and after the storage period and were in the range of 9-12%, showing a slight but not significant decrease during storage. All values were below the limit of 14% established by legislation for safe storage. These values decreased only little during storage (data not shown). Therefore, the moisture decrease can be considered as insignificant and the values found for enzyme activity and concentrations of pigments can be compared disregarding the water content.

Analyses results of water activity were between 0.6 and 0.7 for all samples, a range in which the activity of enzymes is slightly diminished but its activity is still possible.

3.5 Analysis of colorless chlorophyll metabolites

Figure 2a-c shows an example of the results of the HPLC analyses. Analyses after 20 months of storage. The analyses of non-

Table 1. Titratable acidity (%) of soybean samples at different times of storage.

	Titratable acidity (%)				
	BRS 184a	BRS 184b	BRS 133a	BRS 133b	COODETEC 201
at harvest	$1.41^{a} \pm 0.07$	$1.42^{a} \pm 0.24$	$1.32^{a} \pm 0.12$	$1.29^{a} \pm 0.06$	$1.11^{a} \pm 0.21$
20 month	$1.76^{a} \pm 0.47$	$2.76^{b} \pm 0.07$	$2.93^{b} \pm 0.13$	$3.32^{b} \pm 0.15$	$1.84^{b} \pm 0.02$
30 month	$3.42^{b} \pm 0.04$	$3.46^{b} \pm 0.69$	$3.49^{c} \pm 0.03$	$3.92^{c} \pm 0.09$	$2.53^{c} \pm 0.01$

Means with the same letter in the same column are not significantly different at the 0.05 level (Tukey test).

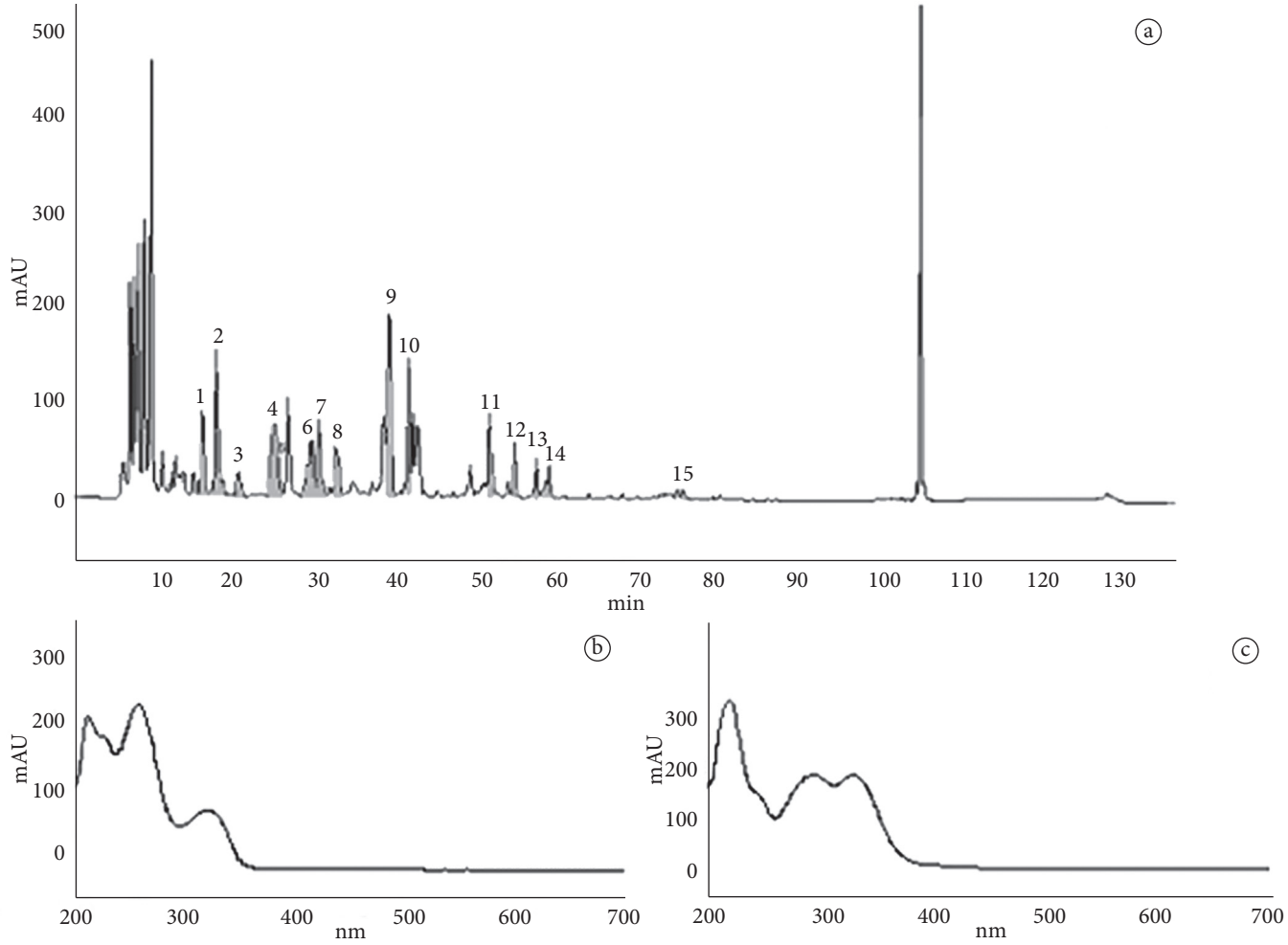

Figure 2. a) Typical HPLC chromatogram of soybean extracts. Numbered peaks presented absorption maxima at 329 nm, except for peak 11, owning an absorption maximum at 320 nm, typical for NCC; b) typical spectrum of peaks with absorption maxima at 218, 295 and 329 nm; and c) spectrum of peak 11 with absorption maxima at 212, 256 e 320 nm.

fluorescent chlorophyll catabolites (NCC) proved the presence of small amounts of colorless chlorophyll catabolites in all varieties.

Characterization was possible due to a comparison of spectra and absorption maxima with those of NCC previously mentioned in the literature (BERGHOLD et al., 2002). Figure 2a shows a chromatogram of the sample COODETEC 201, in which 15 peaks with similar spectra were found. Fourteen of them had spectra with absorption maxima at 329 or 332 nm while only one peak was similar to that reported as characteristic for NCC in the literature with an absorption maximum at 320 nm. Figure 2b and c show examples of the respective absorption spectra. The spectrum in Figure 2b is not recognized in the literature as typical for NCC, but the repetitive presence of fractions with this spectrum is very interesting and needs to be more deeply investigated. The identification of the substances with this profile will be the aim of further studies.

The presence of these derivatives in the seeds even after a long period of storage at room temperature confirms, firstly, that enzymes are still active as NCC are produced as the result of an enzymatic oxygenolytic opening of the chlorophyll macrocycle; secondly, that degradation of chlorophylls and subsequently of NCC is not complete in soybean seeds and that there is a possible accumulation of these colorless substances. NCC are considered to be the final products of chlorophyll degradation stored in the cell vacuoles, as discussed previously in former publications (OBERHUBER et al., 2001). Nevertheless, there have been some doubts about this accumulation since monopyrrolic structures of chlorophyll degradation have already been found (KRÄUTLER, 2006).

4 Conclusions

Drought stress during the maturation period of soybeans caused the retention of green color, and it probably enhanced cell membrane permeability leading to a lower pH. It also promoted non-enzymatic transformation of chlorophylls into pheophytins which were the predominant green-brownish pigments found. After 20 months of storage almost all green pigments had disappeared and seeds had turned yellow presenting an almost normal appearance, except for a smaller size, what seems to be related to drought stress. The changes observed indicate residual enzymes activity, which led to the alteration of some biochemical characteristics.

References

AXELROD, B. et al. Lipoxygenase from soybeans. **Methods in Enzymology,** v. 71, p. 441-451, 1981.

BERGHOLD, J. et al. Chlorophyll breakdown in spinach: On the structure of five nonfluorescent chlorophyll catabolites. **Photosynthesis Research,** v. 74, n. 2, p. 109-119, 2002.

BRASIL. **Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA**. Embrapa Soja. Available from:<http://www.cnpso.embrapa.br>. Access in: January 24th, 2008.

BRASIL. **Ministério da Agricultura, Pecuária e Abastecimento.** Companhia Nacional de Abastecimento. Available from:<http://www.conab.gov.br>. Access in: January 24th, 2008.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. **Resolução n. 11**, de 15 de Maio de 2007. Available from:<http://extranet.agricultura.gov.br/sislegisconsulta/consultarLegislacao.do?operacao=visualizar&id=17751>. Access in: July 28th, 2009.

FEHR, W. R.; CAVINESS, C. E. **Stages of soybean development**. Ames: Iowa State University of Science and Technology, 1977. 11 p.

GOMES, M. S. O. et al. Effect of harvesting and drying conditions on chlorophyll levels of soybean (*Glycine Max* L. Merr). **Journal of Agricultural and Food Chemistry**, n. 51, p. 1634-1639, 2003.

HEATON, J. W.; MARANGONI, A. G. Chlorophyll degradation in processed foods and senescent plant tissues. **Trends in Food Science and Technology,** v. 7, n. 1, p. 8-15, 1996.

HÖRTENSTEINER, S. et al. The key step in chlorophyll breakdown in higher plants: Cleavage of pheophorbide *a* macrocycle by a monooxygenase. **Journal of Biological Chemistry**, v. 273, n. 25, p. 15335-15339, 1998.

HÖRTENSTEINER, S. The loss of green color during chlorophyll degradation: A prerequisite to prevent cell death? **Planta**, v. 219, n. 2, p. 191-194, 2004.

INSTITUTO ADOLFO LUTZ. **Métodos físico-químicos para análise de alimentos.** 4th ed. São Paulo, 2005a. p. 592.

INSTITUTO ADOLFO LUTZ. **Métodos físico-químicos para análise de alimentos.** 4th ed. São Paulo, 2005b. p. 593.

INSTITUTO ADOLFO LUTZ. **Normas analíticas do instituto Adolfo Lutz.** 2nd ed. São Paulo, 1976. v. 1, p. 188-189.

JOHNSTON, M. R.; LIN, R. C. FDA views on the importance of water activity in good manufacturing practice. In: **Water Activity**: Theory and Applications to Food. Chicago: Institute of Food Technologists, 1987.

KRÄUTLER, B. Chlorophyll catabolites and the biochemistry of chlorophyll breakdown. In: **Chlorophylls and bacteriochlorophylls:** Biochemistry, biophysics, functions and applications. [S.l]: Springer, 2006. p . 237-260.

MANGOS, T. J.; BERGER, R. G. Determination of major chlorophyll degradation products. **Zeitschrift für Lebensmitteluntersuchung und Forschung A**, v. 204, n. 5, p. 345-350, 1997.

NOGUEIRA, S. S. S.; NAGAI, V. Effect of simulated water stress during different stages of the early soybean cultivars Paraná. **Bragantia,** v. 47, n. 1, p. 9-14, 1988.

OBERHUBER, M. et al. Chlorophyll breakdown – on a nonfluorescent chlorophyll catabolite from spinach. **Helvetica Chimica Acta**, v. 84, n. 9, p. 2615-2627, 2001.

OLIVEIRA, D. A. et al. Identification of three genotypic classes for soybean lipoxigenases *1* and *3* on enzymatic activity. **Biotechnology Techniques**, v. 12, n. 1, p. 71-74, 1998.

PORRA, R. J. et al. Determination of accurate extinction coefficients and simultaneous equations for assay chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. **Biochimica et Biophysica Acta**, v. 975, n. 3, p. 384-394, 1989.

PRUŽINSKÁ, A. et al. Chlorophyll breakdown: Pheophorbide *a* oxygenase is a Rieske-type iron-sulfur protein, encoded by the *accelerated cell death 1* gene. **Proceedings of the National Academy of Science**, v. 100, n. 25, p. 15259-15264, 2003.

RICE, R. D. et al. Effect of enzyme inactivation on the extracted soybean meal and oil. **Journal of the American Oil Chemists Society**, v. 58, n. 5, p. 578-583, 1981.

ROCA, M. et al. Analysis of the chlorophyll catabolism pathway in leaves of an introgression senescence mutant of *Lolium Temulentum*. **Phytochemistry**, v. 65, n. 9, p.1231-1238, 2004.

SCHWARTZ, S. J.; LORENZO, T. V. Chlorophyll stability during continuous aseptic processing and storage. **Journal of Food Science**, v. 56, n. 4, p. 1059-1062, 1991.

SINNECKER, P. et al. Mechanism of soybean (*Glycine max* L. Merrill) degreening related to maturity stage and postharvest drying temperature. **Postharvest Biology and Technology**, v. 38, n. 3, p. 269-279, 2005.

SOUCI, S. W. et al. **Food composition and nutrition tables.** 7th ed. Stuttgart: Ed. Medpharm Scientific Publishers, 2007. 1364 p.

TAUTORUS, C. L.; LOW, N. H. Chemical aspects of chlorophyll breakdown products and their relevance to canola oil stability. **Journal of the American Oil Chemists' Society**, v. 70, n. 9, p. 843-847, 1993.