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DEPARTAMENTO DE ALIMENTOS E NUTRIÇÃO

Atividade Antioxidante *in vitro* e *in vivo* de suco de uva e da Norbixina

Jane Cristina de Souza

Nutricionista

Profa. Dra. Débora de Queiroz Tavares

Orientadora

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Dissertação apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas para obtenção do título de mestre em Alimentos e Nutrição.

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SUMÁRIO

RESUMO

Estudos epidemiológicos demonstram que o consumo de dietas ricas em alimentos e bebidas de origem vegetal está associado à redução do desenvolvimento de doenças crônico-degenerativas. Tais alimentos são fontes de substâncias como carotenóides e polifenóis que podem atuar como agentes quimioprotetores, reduzindo os danos causados por espécies reativas de oxigênio, formadas tanto em condições fisiológicas quanto patológicas. Os objetivos do trabalho foram determinar *in vivo* a atividade antioxidante do suco de uva e da norbixina contra o estresse oxidativo provocado pela administração de Acetaminofeno (AAP), assim como determinar in vitro, a capacidade antioxidante dos sucos durante o processamento e estocagem. Foram dosados os teores de fenólicos totais pelo método de Folin-Ciocalteau, Catequinas e Epicatequinas por CLAE, principais componentes polares ativos por espectrometria de massa com ionização por electrospray com infusão direta (ESI-MS) e capacidade antioxidante pelo método do DPPH. Para determinação da atividade antioxidante *in vivo* foi conduzido um ensaio biológico com duração de 30 dias. Foram utilizados 30 ratos Wistar machos divididos em 6 grupos (n=5). Os animais ingeriram, duas vezes ao dia, 1 ml de suco de uva Concord (CGJ) (concentração de polifenóis 24mg/mL) ou 1 mL de solução aquosa de Norbixina (Nb) (concentração de 24mg/mL), ou 1 mL de água. Nos 29º e 30º dias os animais receberam intraperitonealmente uma dose de Acetaminofeno (100mg/kg de peso corpóreo). Após o sacrifício foram retirados fígado e rins para análises histológicas e enzimáticas. Os tecidos hepáticos e renais foram analisados por Microscopia Ótica (MO) e Eletrônica de transmissão (MET). Foram dosados os níveis de peroxidação lipídica (TBARS), a atividade das enzimas antioxidantes (SOD, MnSOD, CuZnSOD, GPx, GPx Se-dependente e catalase). Os resultados *in vitro* mostram que os sucos apresentam altos teores de fenólicos totais e capacidade antioxidante, os quais são mantidos durante o processamento e armazenamento do produto. Os resultados *in vivo* mostram que no fígado de animais tratados com CGJ+AAP e Nb+AAP houve diminuição significativa ($p \leq 0.05$) da peroxidação lipídica induzida pelo AAP em 18.7% e 21.0% respectivamente. Por outro lado no rim, a redução foi de 7.1% no grupo CGJ+AAP e 5.3%, no grupo Nb+AAP, valores estes não diferentes ($p \leq 0.05$) em comparação ao grupo AAP. Os níveis de peroxidação lipídica dos grupos que

receberam Suco de uva Concord ou Norbixina, sem a presença de acetaminofeno, não diferiram do grupo Controle ($p \leq 0.05$). O grupo CGJ+AAP mostrou um aumento significativo de 200% no fígado e de 100% nos rins na atividade de catalase em comparação ao grupo AAP. No grupo Nb+AAP a atividade de catalase aumentou 54% no fígado, enquanto que no rim, não ocorreu aumento na atividade de catalase em comparação ao grupo AAP. O estudo demonstra que os sucos analisados apresentam alta capacidade antioxidante a qual foi mantida durante as etapas de processamento e estocagem. Fígado e rins respondem de maneira distinta na presença de antioxidantes, porém ambos CGJ e Nb atenuam a toxicidade causada pelo AAP.

Palavras-chave; atividade antioxidante, suco de uva, norbixina, enzimas antioxidantes, estresse oxidativo.

ABSTRACT

Epidemiological studies shown that the consumption of diets rich in plant foods and beverages is associated with reduction in the development of chronic-degenerative diseases. These foods are sources of substances such as carotenoids and polyphenols that can act as chemoprotectives agents, reducing the damage caused by reactive oxygen species, formed both in physiological and pathological conditions. The objectives of this study were to determine, in vivo, the antioxidant activity of grape juice and Norbixin against oxidative stress induced by Acetaminophen (AAP) administration, as well as determine, in vitro, the antioxidant capacity of juices during the processes of manufacturing and storage. Were determined the total phenolic contents using the Folin - Ciocalteau method; Catechin and Epicatechins by CLAE, major polar components by direct infusion and electrospray ionization mass spectrometry (ESI-MS) and antioxidant capacity by the DPPH method. To the antioxidant activity determination in vivo, was conducted a biological assay with 30 days of duration. They were used 30 rats male Wistar divided in 6 groups (n = 5). The animals were given twice daily 1 ml of Concord grape juice (CGJ) (polyphenols concentration 24mg/mL) or 1 mL of aqueous solution Norbixin (Nb) (concentration 24mg/ mL), or 1 mL of water. In 29 ° and 30 ° days, the animals received a dose of Acetaminophen (100mg/kg of body weight). After sacrifice, liver and kidneys were removed for histological and enzymatic analysis. The liver and kidney tissues were analyzed by optical microscopy (OM) and transmission electronic microscopy (TEM). Were measured lipid peroxidation levels (TBARS), the antioxidant enzymes activity (SOD, MnSOD, CuZnSOD, GPx, GPx Se-dependent and catalase). The *in vitro* results show that juices have high total phenolic levels and antioxidant capacity, which are kept for the processing and storage of these products. *In vivo* results show that liver of animals treated with CGJ+AAP and Nb+AAP was a decrease significant of lipid peroxidation caused by AAP in 18.7% and 20.99% respectively. On the other hand, in the kidney, the decrease was 7.1% in the CGJ+AAP group and 5.3% in the Nb+AAP group, whereas these values were not statistically different ($p \leq 0.05$) compared to the group AAP. Concord grape juice or Norbixin tested alone did not differ from Control group. The CGJ+AAP group showed a significant increase of 200% in the liver and 100% in the kidneys in the catalase activity

when compared to the AAP group. In Nb+AAP group catalase activity in the liver increased 54%, but in the kidney, there was no increase in activity of catalase compared to the group AAP. In this study was verified that juices showed high antioxidant capacity, which is maintained during the stages of processing and storage. Liver and kidneys showed distinct responses in the antioxidants presence, but both CGJ and Nb reduces AAP-toxicity induced.

Keywords: antioxidant activity, grape juice, norbixin, antioxidant enzymes, oxidative stress.

INTRODUÇÃO GERAL

Durante o metabolismo celular podem ser formadas substâncias altamente reativas, derivadas, principalmente do oxigênio molecular e do óxido nítrico. Em condições fisiológicas normais, estas espécies reativas podem ser controladas por mecanismos defensivos celulares, entretanto, em situações de desequilíbrio entre sua produção e a concentração de defesas antioxidantes, estas substâncias podem reagir com os componentes celulares, desencadeando uma série de reações em cadeia que podem culminar com a morte celular (ROVER JUNIOR et al., 2001).

Os danos oxidativos induzidos nas células e tecidos fazem parte da etiologia de várias doenças, incluindo doenças degenerativas tais como as cardiopatias, aterosclerose, câncer e diabetes (RIMM, 2002). Estudos epidemiológicos já demonstraram uma correlação inversa entre o desenvolvimento destas doenças crônico-degenerativas e o consumo de dietas ricas em frutas e vegetais. Os efeitos protetores destes alimentos estão associados à presença de substâncias antioxidantes, especialmente, compostos fenólicos, carotenóides, vitaminas e sais minerais (MANACH et al., 2005).

Polifenóis são os antioxidantes naturais mais ingeridos na dieta humana sendo encontrados nas mais variadas fontes como cereais, chocolates, chás, café, frutas e produtos derivados, como vinhos e sucos (SCALBERT, JOHNSON & SALTMARSH, 2005). O Suco de uva é importante fonte de compostos polifenólicos, principalmente catequinas, epicatequinas, quercetinas, antocianinas, e proantocianidinas, cujos teores podem variar de acordo com o tipo de processamento e condições de estocagem (RIBEREAU-GAYON & PEYNAUD, 1971; FULLEKI & RICARDO-DA-SILVA, 2003). Os estudos comprovam que, em humanos, o consumo de sucos de uva promove proteção contra a oxidação da lipoproteína de baixa densidade, diminuição da agregação plaquetária e aderência endotelial (O' BYRNE, 2002).

Os carotenóides compõem outra conhecida classe de antioxidantes naturais que podem atuar como seqüestradores de radicais livres inibindo a propagação da reação em cadeia tanto *in vitro* quanto *in vivo* (VANDENBERG, et al., 2000). A norbixina é uma substância carotenóide encontrada nas sementes da *Bixa orellana L* (urucum), a qual tem uso permitido como corante alimentar em muitos países (KOVARY et al., 2001). Estudos demonstram que a norbixina possui grande potencial antioxidante por ser capaz de

seqüestrar os radicais superóxido e neutralizar o oxigênio singlete (DI MASCIO et al., 1990; ZHAO el at.,1998). Além do mais, pesquisas posteriores comprovaram que a norbixina pode induzir a atividade das monoxigenases do sistema citocromo P450, responsáveis pelas reações de fase I de detoxificação hepática (JEWELL & O'BRIEN, 1999; DE-OLIVEIRA, et al., 2003).

Baseado no exposto acima, os objetivos deste trabalho foram determinar através de análises *in vivo* a atividade antioxidante do suco de uva e da norbixina, assim como determinar *in vitro*, a capacidade antioxidante de sucos de uva durante os processos de fabricação e estocagem.

A presente dissertação está dividida em cinco capítulos. Os resultados estão apresentados na forma de artigos científicos, que se encontram nos capítulos 2, 3 e 4. Os capítulos 2 e 3 apresentam dados de qualificação tecnológica do suco de uva, assim como a respectiva capacidade antioxidante do produto *in vitro*, durante o processamento e estocagem. O capítulo 4 apresenta a comparação da atividade antioxidante, *in vivo*, do suco de uva e da norbixina. Ao final, o capítulo 5 apresenta a conclusão geral da pesquisa.

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CAPÍTULO 1: REVISÃO DE LITERATURA

1. Radicais livres

O termo radical livre é definido como qualquer átomo ou molécula que apresente um ou mais elétrons desemparelhados em sua órbita externa. Esta configuração faz dos radicais livres moléculas altamente instáveis, de meia-vida curta e quimicamente muito reativas (HALLIWELL & GUTTERIDGE, 1990).

Os radicais livres são formados durante processos fisiológicos de oxidação, podendo ser produzidos no citoplasma, nas mitocôndrias e nas membranas celulares (HALLIWELL & GUTTERIDGE, 1998; BARREIROS, 2006).

Em condições fisiológicas, os radicais livres participam dos processos de fagocitose, de sinalização celular e também estão envolvidos na síntese de algumas proteínas (HALLIWELL & GUTTERIDGE, 1998). Por outro lado, podem provocar reações em cadeia causando danos a um grande número de moléculas.

Na natureza existem duas importantes substâncias geradoras de radicais livres: o oxigênio no estado fundamental (O_2) e o óxido nítrico (NO) (ROVER JUNIOR et al., 2001). Durante o metabolismo, tais substâncias podem gerar componentes altamente reativos denominados espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN).

1.1 Espécies reativas de oxigênio (ERO)

A formação das ERO foi inicialmente descrita por McCORD & FRIDOVICH (1968), quando demonstraram a formação do radical $O_2^{\bullet -}$ (ânion superóxido), pela ação da enzima xantina oxidase. Posteriormente, McCORD (1974), demonstrou a capacidade das ERO em causar danos oxidativos aos tecidos (McCORD, 1974).

Embora o oxigênio seja um elemento vital para os organismos aeróbios, permitindo a utilização da energia proveniente de nutrientes pelo processo de fosforilação oxidativa, uma pequena fração do seu consumo mitocondrial é transformada em espécies reativas de oxigênio (VANNUCCHI, 1998).

É conhecido que durante a fosforilação oxidativa, ocorre a redução tetravalente do oxigênio molecular, através da aquisição de 4 elétrons com conseqüente produção de 2

moléculas de água. Neste processo são gerados intermediários e cerca de 2–5% do O_2 pode dar origem à formação de EROs como descrito abaixo:

$$O_2 + e^- \rightarrow O_2^{\bullet -}$$

$$O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + e^- \rightarrow HO^- + HO^{\bullet}$$

$$HO^{\bullet} + e^- + H^+ \rightarrow H_2O$$

$$\overline{O_2 + 4\,e^- + 4H^+ \rightarrow 2H_2O}$$

Equação 1: Redução tetravalente do oxigênio molecular.

Entre as espécies reativas de oxigênio mais comuns, podemos citar o radical superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($HO^{\bullet}$) e o oxigênio singlete ($^1O_2$) (SILVEIRA, 2004).

- Radical superóxido ($O_2^{\bullet -}$)

O radical superóxido é a ERO mais comum e abundante nas células aeróbicas (BOVERIS, 1998). Nestes organismos, o $O_2^{\bullet -}$ é formado, sobretudo através da cadeia de transporte de elétrons ou pela ação de células fagocitárias como neutrófilos, monócitos e macrófagos, durante a atividade bactericida (DIAZ *et al*., 1998).

As células fagocitárias produzem quantidades significativas do radical superóxido, durante a fagocitose, devido à ativação da enzima NADPH oxidase presente em suas membranas (OGA, 2003), conforme expresso na equação abaixo:

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet -} + NADP^+ + H^+$$

Equação 2 - Formação de radical superóxido.

Foi verificado que a atuação do radical superóxido ($O_2^{\bullet -}$) como oxidante direto é irrelevante. Seu potencial tóxico está relacionado à sua capacidade de gerar outras espécies de maior reatividade, como o radical hidroxila e o peroxinitrito (MISRA & FRIDOVICH, 1972; BARREIROS, 2006).

- Peróxido de hidrogênio (H_2O_2)

O peróxido de hidrogênio é formado, sobretudo na matriz mitocondrial, durante o processo de redução do oxigênio, ou pela ação das superóxido dismutases na redução do radical superóxido (FRIDOVICH, 1998).

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Equação 3 – Formação de peróxido de hidrogênio.

Apesar de não ser considerado um radical livre, pela ausência de elétrons desemparelhados na última camada, o H_2O_2 é um metabólito do oxigênio extremamente deletério, devido a sua participação na reação que produz o radical hidroxila ($HO^{\bullet}$), espécie reativa de oxigênio extremamente tóxica (FERREIRA & MATSUBARA, 1997).

- Radical hidroxila ($HO^{\bullet}$)

Dentre as ERO, o radical hidroxila ($HO^{\bullet}$) é considerado o mais potente oxidante em sistemas biológicos devido a sua alta reatividade apesar de seu tempo de vida extremamente curto ($1x10^{-9}$s). A ação do radical ($HO^{\bullet}$) é rápida e inespecífica atingindo alvos celulares mais próximos, podendo causar danos ao DNA, proteínas, carboidratos e lipídios. (HALLIWELL & GUTTERIDGE, 1986).

Os radicais ($HO^{\bullet}$) podem ser gerados na cadeia de transporte de elétrons e, além disso, outras duas diferentes vias estão envolvidas em sua produção: a reação de Fenton e a de Haber-Weiss, descritas à seguir:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^-$$ (reação de Fenton) (4).

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe} O_2 + HO^{\bullet} + OH^-$$ (reação de Haber-Weiss) (5).

A presença de íons de metais de transição funciona como um dos fatores de promoção da formação de radicais livres. O ferro é o metal mais abundante no organismo humano e o principal catalisador nas reações de oxidação de biomoléculas. Além do ferro,

o cobre também participa de processos oxidativos e pode catalisar a reação de Haber-Weiss (VANNUCCHI, 1998; BARREIROS, 2006).

- Oxigênio Singlete ($^1O_2$)

Assim como o H_2O_2 o Oxigênio Singlete não é considerado um radical livre, pela ausência de elétrons desemparelhados em sua última camada eletrônica. O oxigênio singlete é caracterizado como uma forma excitada do oxigênio molecular a qual pode ser gerada, por exemplo, por fagócitos ou por indução luminosa ou durante o processo de peroxidação lipídica (HALLIWELL, 1996).

1. 2 Espécies reativas de nitrogênio (ERN)

- Radical oxido nítrico ($NO^{\bullet}$)

Outra importante espécie reativa é o óxido nítrico ($NO^{\bullet}$), produzido no organismo pela ação da oxido nítrico sintetase a partir do aminoácido L-arginina (MARLETTA, et al., 1988). Assim como o radical superóxido, seu potencial tóxico está relacionado à formação de outras espécies de grande reatividade como o radical hidroxila e o peroxinitrito, conforme representa a equação 6:

$$O_2 + NO^{\bullet} \rightarrow ONOO^-$$

Equação 6: Formação de Peroxinitrito à partir do radical óxido nítrico.

- Peroxinitrito ($ONOO^-$)

O peroxinitrito é considerado um agente oxidante com grande potencial citotóxico, podendo agir diretamente sobre moléculas biológicas através da oxidação de grupamentos sulfidrilas (HALLIWELL & GUTTERIDGE, 1995; SZABO, 2003). Durante o processo de decomposição do peroxinitrito outras espécies reativas são formadas, incluindo o radical hidroxila, conforme abaixo:

$$ONOO^- + H^+ \rightarrow HO^{\bullet} + NO_2^{\bullet}$$

Equação 7 – Processo de decomposição do peroxinitrito com formação de novas espécies reativas de oxigênio.

2 Antioxidantes

Antioxidantes são definidos como substâncias que presentes, em baixas concentrações em relação ao substrato oxidável, retardam ou previnem a oxidação deste substrato. Assim, os antioxidantes atuam como protetores da oxidação de biomoléculas por radicais livres e impedem a propagação da reação em cadeia provocada pelos mesmos (HALLIWELL & GUTTERIDGE, 1998; FANG et al., 2002).

Os sistemas antioxidantes podem ser divididos em dois tipos: enzimáticos e não enzimáticos (NORDBERG & ARNER, 2001).

2.1 Sistemas de defesas enzimáticos

O sistema de defesa antioxidante enzimático inclui a atividade das enzimas: (1) superóxido dismutase (SOD), (2) catalase (CAT), (3) glutationa peroxidase (GPx), (4) glutationa redutase (GR) (SIES, 1993; BONNEFOY et al., 2002). Existem outros sistemas que participam na remoção do excesso de espécies reativas de oxigênio, tais como a (1) glutationa S-transferase (GST), as (2) superóxido redutases, as (3) peroxirredoxinas e o sistema associado à (4) tiorredoxina (composto por duas oxidorredutases antioxidantes) (NORDBERG & ARNER, 2001).

2.1.1 Superóxido dismutase (SOD)

As superóxido dismutases constituem a primeira linha de defesa enzimática contra a produção intracelular de radicais livres, catalisando a dismutação do radical superóxido a peróxido de hidrogênio (HALLIWELL & GUTTERIDGE, 1999; HOLLANDER et al., 2000).

Os sistemas eucariontes exibem duas formas de SOD. A forma CuZnSOD, presente principalmente no citosol e meio extraceluar, e a MnSOD localizada na mitocôndria (FERREIRA & MATSUBARA, 1997).

2.1.2 Catalase (CAT)

A catalase é uma ferrihemoenzima, localizada, sobretudo nos peroxissomas, cuja principal função é converter o peróxido de hidrogênio formando água e oxigênio molecular

(FRIDOVICH, 1998). Na reação, uma das moléculas de peróxido de hidrogênio é oxidada a oxigênio molecular e a segunda é reduzida à água (CHANCE et al., 1979).

A catálise do peróxido de hidrogênio é de extrema importância para a célula, pois, na presença de Fe^{+2}, ocorre a formação do radical hidroxila (reação de Fenton), altamente reativo e danoso às biomoléculas (ARRIGONI & DE TULLIO, 2002; MATOS, et al., 2006).

2.1.3 Glutationa peroxidase (GPx) e glutationa redutase (GR)

A glutationa peroxidase está presente tanto no citosol (forma selênio-dependente) quanto na matriz mitocondrial (selênio-independente); sua função é catalisar a redução do peróxido de hidrogênio e hidroperóxidos orgânicos para água e álcool, usando a glutationa (GSH) como doadora de elétrons (HALLIWELL & GUTTERIDGE, 1998).

A GSH-Rd é uma flavoproteína dependente da nicotinamida-adenina-dinucleotídeo-fosfato reduzida (NADPH) e, portanto, também dependente da integridade da via das pentoses (ROVER JUNIOR, 2001). É a enzima responsável pela manutenção das concentrações intracelulares de GSH através da redução da glutationa oxidada (GSSG) (DAMIANI, 2006).

A figura 1 ilustra o sistema de defesa antioxidante enzimático, explicitado acima.

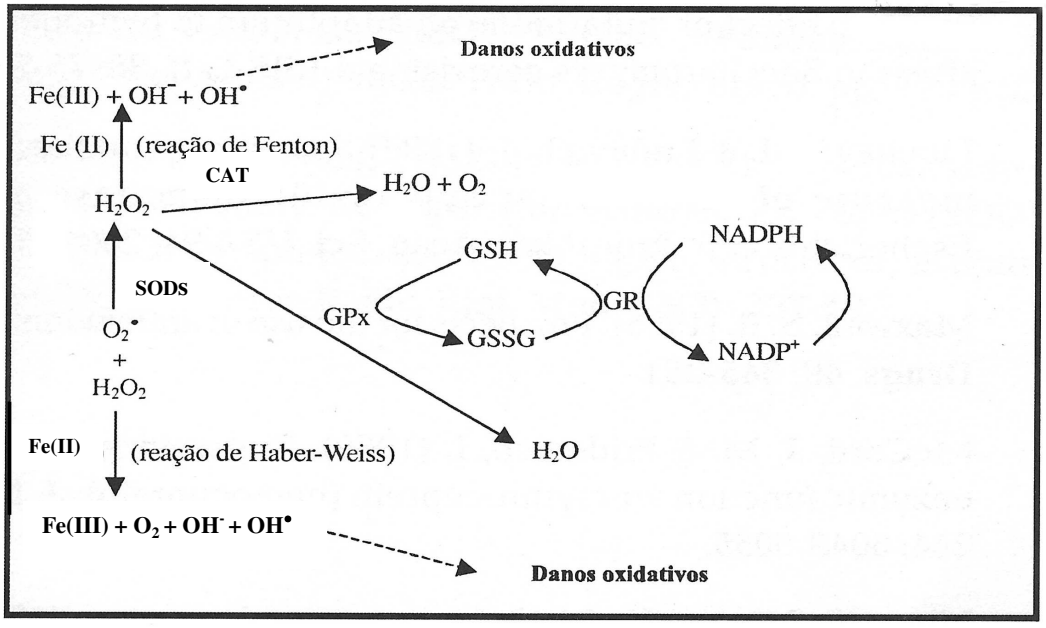

Figura 1 – Sistema de defesa antioxidante enzimático.

2.2 Antioxidantes Não-Enzimáticos

O sistema de defesa antioxidante não enzimático é composto por moléculas com capacidade de proteger determinados alvos biológicos contra a oxidação. Tais moléculas podem ter origem endógena ou podem ser obtidas através da dieta (SIES, 1993).

Para serem consideradas antioxidantes, as substâncias têm que apresentar pelo menos uma das três propriedades: supressão da formação de radicais livres (por quelação de metais ou por inibição de enzimas geradoras de radicais livres), eliminação ou desativação de radicais livres com formação de um produto estável, ou participação em processos de reparo de danos oxidativos (BOURNE & RICE-EVANS, 1999; RIBEIRO, 2005).

Dentre os antioxidantes endógenos a glutationa (GSH), um tripeptídio γ-L-glutamil-L-cisteinil-glicina, exerce importante papel no sistema de defesa antioxidante (MEISTER, 1983). Presente na maioria das células, a GSH tem sua capacidade redutora determinada pelo grupamento sulfidrila da cisteína. Após exposição da GSH ao agente oxidante, ocorre sua oxidação a GSSG. A recuperação da GSH é feita pela enzima GSH-Rd, uma etapa essencial para manter íntegro o sistema de proteção celular (GILBERT, 1990; GASPARRI, 2005).

Dentre os antioxidantes obtidos pela dieta, estão incluídas micromoléculas lipofílicas e hidrofílicas, com capacidade de atuar em compartimentos biológicos, apolares e polares, respectivamente. As principais substâncias deste grupo são: tocoferol, o ascorbato, os carotenóides e os compostos fenólicos (HUANG, 2005; BARREIROS, 2006).

2.2.1 Compostos Fenólicos

Os polifenóis constituem uma classe de substâncias representada por mais de 8 mil compostos diferentes. Considerados metabólitos secundários das plantas são produzidos em resposta a agentes agressores como a radiação ultravioleta ou patógenos (MARTINEZ-VALVERDE et al., 2000).

Quimicamente são substâncias que possuem em sua estrutura anéis benzênicos associados a grupamentos hidroxilas, em diferentes posições (CHEYNIER, 2005). De acordo com o número de anéis fenólicos e com os elementos ligados a estes anéis os

compostos fenólicos se dividem em grupos: (1) ácidos fenólicos, (2) flavonóides, (3) estilbenos e (4) ligninas (MANACH et al, 2004).

Os ácidos fenólicos por sua vez se dividem em dois grupos: (a) os derivados do ácido benzóico, presentes em maiores quantidades nas frutas vermelhas e chás, sendo o ácido gálico o principal representante; e os (b) derivados do ácido hidroxicinâmico encontrados, sobretudo nas partes mais externas e na casca de frutas (SOARES, 2002).

Os flavonóides são constituídos quimicamente por dois anéis aromáticos ligados por três átomos de carbono que formam um heterociclo oxigenado (figura 2). De acordo com os tipos de funções presentes no heterociclo podem se dividir em 6 subclasses: (a) flavonóis, (b) flavonas, (c) flavanonas, (d) isoflavonas, (e) antocianidinas e (f) flavanols (proantocianidinas e catequinas) (MANACH et al, 2004).

Os estilbenos são encontrados especialmente nas uvas, sendo o resveratrol o principal representante da classe (VINTRAC, et al., 2002). As ligninas são polímeros complexos de grande resistência mecânica, cuja hidrólise alcalina libera uma grande variedade de ácidos benzóico e cinâmico (HEINONEN, et al., 2001).

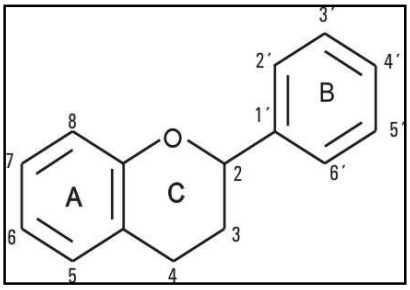

Figura 2- Estrutura básica de um composto flavonóide.

Os polifenóis atuam como seqüestradores de radicais livres e íons metálicos causando modificações no estado de *redox* da celular e desencadeando um conjunto de reações dose-dependentes (MANACH et al, 2004). Pesquisas apontam que os polifenóis também podem interagir com receptores e/ou enzimas relacionadas com os sinais de transdução intracelulares (WILLIAMS, SPENCER & RICE-EVANS, 2004; SOUSA, et al., 2007).

Os polifenóis são os antioxidantes mais ingeridos na dieta de humanos podendo atingir níveis em torno de 1g/dia, sobretudo nas populações de regiões tropicais e subtropicais. Diversos estudos sugerem que a prevenção de doenças degenerativas, sobretudo cardiovasculares e cânceres, estão associadas à ação antioxidativa dos compostos fenólicos contidos nos alimentos (MANACH et al., 2004).

Pesquisas também mostram que a absorção intestinal de polifenóis ocorre em quantidades diferenciadas e dependem da matriz de nutrientes gerada no lúmen intestinal (HALLIWELL et al., 2005). O conjunto de metabólitos polifenólicos ao atingirem a corrente sanguínea, ligam-se às proteínas plasmáticas, sobretudo à albumina, principal responsável pelo transporte destas substâncias (HE & KIES, 1994; O' BYRNE et al., 2002).

A retenção de polifenóis em tecidos corporais ocorre de maneira diferenciada ainda não claramente explicada pela literatura, porém já se sabe que a quantidade de metabólitos fenólicos tende a ser maior nos vários tecidos corporais do que no plasma sanguíneo (MANACH et al., 2004).

Estudos recentes mostram que compostos fenólicos das mais diversas fontes reduziram os níveis de peroxidação lipídica em tecidos, como fígado, intestinos, rins e cérebro, e também demonstraram capacidade de aumentar a atividade antioxidante enzimática (JODYNIS-LIEBERT et al., 2005; OJO, et al., 2006; KASDALLAH-GRISSA, et al., 2007).

2.2.1.1 Compostos fenólicos presentes no suco de uva

O conteúdo fenólico das uvas varia de acordo com a espécie, variedade, maturidade, condições climáticas e cultivar (MAZZA, 1995; MANACH et al, 2004). Determinados tratamentos aos quais a uva e o mosto são submetidos durante a produção do suco tais como tipo de extração, tempo de contato entre o suco e as partes sólidas da uva (casca e sementes), prensagem, tratamentos térmicos e enzimáticos, adição de dióxido de enxofre e ácido tartárico, também interferem na quantidade destes compostos no suco pronto (SISTRUNK & GASCOIGNE, 1983; FRANKEL, et al., 1998).

Os principais compostos fenólicos encontrados no suco de uva são os flavanols (catequinas e epicatequinas), as antocianinas, a quercetina e o kaemferol (RIBEREAU-GAYON & PEYNAUD, 1971; FULLEKI & RICARDO-DA-SILVA, 2003).

Diversos trabalhos vêm sendo realizados para comprovar a capacidade antioxidante dos sucos de uva. Resultados de experimentos *in vitro* mostram que em sucos de uvas vermelhas, a atividade antioxidante é similar à encontrada em vinhos tintos (FRANKEL, et al., 1998; BERMÚDEZ-SOTO & TOMÁS-BERBERÁN, 2004).

Quanto a biodisponibilidade dos polifenóis, sabe-se que é depende da forma como o composto se encontra na matriz alimentar (ligação com açúcares, polimerização, etc). Interações com outros alimentos e antioxidantes no trato gastrintestinal também influenciam o aproveitamento destes compostos (SCALBERT & WILLIAMSON, 2000; MANACH et al., 2004).

Sucos de uva contêm um maior teor de compostos fenólicos glicosilados em relação aos vinhos (SINGLETON, 1987) e, segundo HOLLMAN et al. (1995), nesta forma podem ser mais facilmente absorvidos pelo organismo do que suas respectivas agliconas.

Estudos mostram que extratos e sucos de uva, especialmente uvas vermelhas, apresentam grande potencial benéfico à saúde (FRANKEL, 1999). Suco de uva Concord inibiu a oxidação de LDL apresentando atividade antioxidante comparável aos vinhos tintos californianos (FRANKEL, 1998; MANACH, et al., 2005). Pesquisas com humanos demonstraram a capacidade do suco de uva em inibir a iniciação do processo aterosclerótico por reduzir a oxidação da LDL e por inibir a agregação plaquetária (STEIN et al., 1999; CHOU, et al., 2001).

A suplementação com 10 ml/ kg/ dia de suco de uva Concord (560 mg de equivalentes fenólicos/ L) apresentou maior efeito protetor da oxidação de proteínas celulares em relação ao α-tocoferol (400 UI/ dia) (O' BYRNE, 2002). Indivíduos fumantes (19-57 anos) suplementados com 480 mL de suco de uva/dia durante oito semanas, apresentaram uma redução de 25% no dano oxidativo ao DNA linfocitário (PARK et al., 2003).

Em um estudo com ratas portadoras de câncer de mama, CHEN et al. (1998) mostram a redução do volume tumoral em 75% em ratas suplementadas com 0,5 ml/ suco de uva dia.

Ainda em relação aos efeitos benéficos do suco de uva à saúde, SHUKITT-HALE et al. (2006) mostram que ratos suplementados com este produto apresentam menor déficit motor e cognitivo durante o envelhecimento (SHUKITT-HALE et al., 2006).

2.2.2 Carotenóides

Na natureza, a família dos carotenóides é composta por mais de 600 tipos de pigmentos, responsáveis pela coloração, amarela, laranja e vermelha de grande número de frutas, folhas e flores (BOBBIO, 2001).

Quimicamente os carotenóides podem ser classificados em dois grandes grupos: aqueles que contêm apenas átomos de carbono e hidrogênio designados carotenos e aqueles que contêm pelo menos um átomo de oxigênio, compondo grupos ceto-hidroxi ou epóxi-carotenóides, que recebem o nome de xantofilas (VAN DEN BERG, 2000). Parte dos carotenóides são acíclicos, como o licopeno, porém a grande maioria contém um ou dois anéis na molécula, como o β-caroteno (MATTOS, 2001).

A estrutura química dos carotenóides é o elemento responsável pelo seu potencial antioxidante, o qual está relacionado à presença de duplas ligações conjugadas, as quais tornam possível a captação de radicais livres. Além disso, os tipos de grupamentos terminais (cíclicos ou acíclicos) e a natureza dos substituintes em carotenóides de cadeias cíclicas, influenciam a ação supressora de radicais livres destes compostos (HIRAYAMA et al., 1994, KIOKIAS, 2004).

A atividade antioxidante, *in vitro*, dos carotenóides foi bem estabelecida pela descrição de suas propriedades de supressão do oxigênio singlete (FOOTE et al., 1968) e sua habilidade em seqüestrar radicais peroxilas (BURTUN et al., 1984).

Em sistemas biológicos, os carotenóides são capazes de captar energia do oxigênio singlete, que volta ao estado fundamental (O_2). O carotenóide excitado resultante libera baixa energia sendo, nesta situação, inofensivo ao meio celular. Contudo, os diferentes mecanismos sob os quais os carotenóides podem captar espécies reativas levam a uma

variedade de radicais carotenóides e, portanto a múltiplos produtos finais. O potencial protetor ou deletério destes produtos finais depende da natureza do radical, de seu meio ambiente (aquoso ou lipídico) e de características estruturais, como terminal cíclico ou acíclico, grupos finais polares ou apolares, propriedades redox, etc (CERQUEIRA, MEDEIROS & AUGUSTO, 2007).

Experimentos recentes mostraram que em concentrações elevadas a capacidade antioxidante de carotenóides se encontra diminuída, e que neste caso licopeno, luteína e β-caroteno apresentaram efeitos pró-oxidantes (EL-AGAMEI, 2004; KIOKIAS, 2004).

2.2.2.1 Norbixina

Dentre os carotenóides naturais a bixina, principal pigmento do corante urucum (Bixa orellana), é uma substância lipossolúvel a qual pode ser transformada industrialmente, por remoção de um grupamento metil-éster, em um pigmento hidrossolúvel denominado norbixina. (SCOTTER et al., 2002).

Na última década os pesquisadores afirmam a capacidade da bixina/norbixina em aniquilar superóxidos, peroxinitritos e radicais hidroxilas (SANTOS et al., 2002). Alguns estudos demonstraram que, dentre os carotenóides naturais, a bixina é um dos mais ativos seqüestradores de oxigênio singlete ($^1O_2$).

Pesquisas sobre o metabolismo da bixina/norbixina demonstram a capacidade de proteger o tecido hepático da peroxidação lipídica além da capacidade de regular os níveis de LDL e HDL (ZHAO et al.,1998; SANTOS et al., 2002). Outros estudos mostram a capacidade da norbixina em induzir a atividade das monoxigenases do sistema citocromo P450, responsáveis pelas reações de fase I durante a metabolização hepática de xenobióticos (JEWELL & O'BRIEN, 1999; DE-OLIVEIRA, 2003).

A quantificação da bixina/norbixina proveniente da dieta, ainda é discutida sendo que a maioria dos trabalhos não têm demonstrado correlação significativa entre quantidade ingerida e concentração tissular e/ou atividade antioxidante dose dependente (NARISAWA et al., 1996; PAUMGARTTEN et al., 2002). Finalmente, em relação à distribuição tecidual, PAUMGARTTEN e colaboradores citam que fígado, rins e sangue parecem ser os tecidos alvos de retenção da norbixina (PAUMGARTTEN et al., 2002).

3. Estresse oxidativo

Em condições fisiológicas normais, os radicais livres são formados em proporções que podem ser controladas pelos mecanismos defensivos celulares. A falta de equilíbrio dinâmico entre a produção de oxidantes e a concentração de defesas antioxidantes, levam ao chamado estresse oxidativo (CERQUEIRA, MEDEIROS & AUGUSTO, 2007).

O estresse oxidativo pode resultar de situações onde ocorram diminuição nos níveis das enzimas antioxidantes, elevação na produção de radicais livres, ou por ambos os processos simultaneamente. (BONDY & LE BEL, 1993; CADENAS & DAVIES, 2000).

No estresse oxidativo, as ERO e as ERN promovem reações com substratos biológicos, podendo ocasionar danos às biomoléculas. Os danos mais graves são aqueles causados às cadeias de DNA e RNA. O RNA citoplasmático e o DNA mitocondrial são dois dos alvos mais sensíveis ao ataque oxidativo (MIRANDA et al., 2000).

Diversas alterações (e.g. clivagem do DNA, ligações DNA-proteínas, oxidação das purinas, etc.) podem ser causadas por espécies reativas de oxigênio, em especial pelo radical hidroxila. O DNA mitocondrial é o primeiro alvo de ataques oxidativos, devido à sua proximidade com a fonte de produção de ERO (SHARMA & MORGAN, 2001). Estudos recentes demonstraram ainda que os danos causados no DNA mitocondrial são amplificados pela presença de ferro no interior da célula (EATON & QIAN, 2002, VALKO, 2005).

Uma vez quebrada, a cadeia de DNA pode ser reconectada em diferentes posições, o que pode ocasionar mutações celulares devido ao emparelhamento desordenado das bases nitrogenadas (NORDBERG & ARNER, 2001). O acúmulo destas lesões no DNA está relacionado aos processos de mutagênese, carcinogênese e envelhecimento (RIBEIRO et al., 2005).

Outros alvos celulares suscetíveis a danos oxidativos são as proteínas. A ação das ERO na estrutura protéica leva a fragmentação das cadeias polipeptídicas, a formação de ligações proteína-proteína e a modificações nos aminoácidos das cadeias laterais (FAGAN et al., 1999). Como conseqüências destas reações, podem ocorrer perdas na atividade enzimática, dificuldades no processo de transporte ativo, citólise e morte celular (CHOI et al., 2003).

Além do DNA e das proteínas, os lipídeos de membrana são excelentes alvos de ataque por parte das espécies reativas, devido a sua estrutura química contendo múltiplas duplas ligações, o que desencadeia o processo denominado peroxidação lipídica (NORDBERG & ARNER, 2001).

O processo de peroxidação lipídica pode ser distinguido em três passos: iniciação, propagação e terminação. A peroxidação pode ser induzida por radicais suficientemente reativos para remover um átomo de hidrogênio dos ácidos graxos poli-insaturados. Este é o ponto de partida de um ciclo de propagação que leva ao aumento da produção de radicais livres e ao aumento de hidroperóxidos lipídicos formados pela oxidação de inúmeras moléculas lipídicas. Este ciclo de propagação é quebrado quando dois radicais se juntam, formando não-radicais, nas chamadas reações de terminação (LEHUÉDÉ et al., 1999; SPITELLER, 2001).

Como conseqüências do processo de lipoperoxidação ocorrem perdas na permeabilidade seletiva das membranas, liberação do conteúdo de organelas, como as enzimas hidrolíticas dos lisossomas, e formação de produtos citotóxicos como o malonaldeído, culminando com a morte celular (FERREIRA & MATSUBARA, 1997).

Devido a todas estas alterações nas estruturas celulares, o estresse oxidativo é considerado um evento celular de extrema importância sendo sua presença associada ao surgimento e a progressão de diversos processos patológicos, como, por exemplo, câncer, ateriosclerose, diabetes, mal de Alzheimer (DROGE, 2003; EL-AGAMEY, 2004; LANKIN, 2005; VALKO, 2006).

3.1 Acetaminofeno (AAP) como fator de injúria oxidativa

O Acetaminofeno (paracetamol, 4'-hidroxi-acetanilida, N-acetil-p-aminofenol ou 4-acetaminofenol), medicamento utilizado mundialmente como analgésico e antipirético, é considerado seguro pela FDA sob condições normais de uso (MIROCHNITHENKO et al., 1999). Entretanto, com a utilização de grandes doses (overdoses) a droga apresenta alto grau de toxicidade, podendo levar a falhas agudas em órgãos, especialmente fígado, rins e sistema nervoso central (MITCHELL, 1973; COLBDEN, 1982).

O fígado, sítio primário de detoxificação, é o órgão mais acentuadamente atingido pela toxicidade da droga. Sob condições normais de uso, o acetaminofeno (AAP) é metabolizado por sulfatação e glicuronidação hepática e uma pequena parcela sofre transformação pelo sistema de oxidases do citocromo P450 (BESSEMS &VERMEULE, 2001).

O AAP, ao ser metabolizado pelo citocromo P450, produz um intermediário altamente reativo denominado N-acetil-p-benzoquinoneimina (NPQI), o qual pode ser detoxificado com a utilização da glutationa (GSH) (ALBANO et al., 1985).

Quando o AAP é utilizado em doses tóxicas as três rotas de metabolização acima citadas podem ser saturadas levando a lesões teciduais. A forma principal pela qual a droga causa danos celulares ainda não é completamente estabelecida, porém dois mecanismos são bem explicitados por grande número trabalhos realizados nas duas últimas décadas (GIBSON, 1996).

O primeiro mecanismo, conhecido como teoria da ligação covalente, mostra a ligação do NPQI a macromoléculas celulares resultando em injúria tecidual (BIRGE, 1991; SALMINEN, 1998).

O segundo, chamado de teoria do estresse oxidativo, mostra a capacidade do NPQI em oxidar e depletar os níveis celulares de glutationa, o que leva a uma redução no índice GSH/GSSG, caracterizando um quadro de estresse oxidativo (NELSON, 1995; GIBSON, 1996).

O mecanismo oxidativo também é sustentado pela via de redução do NPQI pelo NADPH, com concomitante redução do oxigênio molecular a ânion superóxido, o qual por sua vez poderá ser convertido enzimaticamente a radicais hidroxila que dão início ao processo de peroxidação lipídica (YOUNES, 1986; GOEPTAR,1995).

Com base nestes mecanismos de detoxificação, o acetaminofeno se tornou uma das drogas mais utilizadas na pesquisa bioquímica e toxicológica. Atualmente grande número de substâncias tem seu potencial quimioprotetor testado contra o uso do AAP (BESSEMS & VERMEULE, 2001).

Entre os compostos testados se destacam aqueles com capacidade de seqüestrar radicais livres como, o β-caroteno, o α-tocoferol (LEWERENZ et al., 2003) e os polifenóis das mais diversas fontes (JODYNIS-LIEBERT et al., 2005; OJO et al., 2006). Substâncias potencialmente capazes de aumentar os níveis de GSH, como a cisteína (MANAUTOU et al., 1996) e taurina (WATERS et al., 2001) também vem sendo testadas no controle ao estresse oxidativo causado pelo AAP**.**

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CAPÍTULO 2: ARTIGO DE PESQUISA:

Processing and Storage of Concentrated Grape Juices: Radical Scavenging Activity and Major Active polar Components.

(Artigo submetido ao Journal of Agricultural and Food Chemistry).

Processing and Storage of Concentrated Grape Juices: Radical Scavenging Activity and Major Active polar Components.

Abstract:

The total phenolic (TP) and radical scavenging activity (RSA) of concentrated grape juices during process and storage have been characterized and quantified. TP was determined using the Folin-Ciocalteu method and RSA by the DPPH assay. Main components of juices were investigated by direct infusion electrospray ionization mass spectrometry (ESI-MS) in the negative ion mode. Concord juice (CJ) demonstrated higher RSA and TP contents than Isabel juice (IJ) with some differences at each processing step. During the storage, retention of TP and RSA was 90% e 77% in CJ and of 81% and 86% in IJ, respectively. During processing, peonidin and peonidin-3-O-glucoside were replaced by malvidin and dimethoxy-flavylium as significant components; malvidin and piceatanol-O-glucoside decreased after 8-month storage. Concentrated and refrigerated storage were effective in preserving total phenolics and antioxidative status of grape juices.

Key-words: Concord grape; Isabel grape; total phenols; antioxidant activity; radical scavenging activity; DPPH; concentration, ESI-MS fingerprinting.

1- Introduction:

Phenolic-rich foods have received increasing attention due to recent findings on its association with disease prevention (ARTS & HOLLMAN, 2005; KNEKT et al., 2002; SESSO, et al., 2003). Studies have identified polyphenol dietary sources as mainly being fruits, fruit juices and beverages such as wine, tea, coffee (BEECHER, 2003; BRAVO, 1998; SCALBERT, JOHNSON & SALTMARSH, 2005; SCALBERT & WILLIAMSON, 2000). Average daily intake has been difficult to estimate for reasons mostly related to polyphenol structural diversity and variation of content in particular foodstuffs influenced by cultivar and fabrication processes (SCALBERT et al., 2000). In most studies addressing food composition, some of the new compounds formed during processing and storage of fruit beverages are often overlooked, although they might show particular properties different from their precursors (CHEYNIER, 2005).

The efficacy of natural antioxidants is related to the protection of the food itself against oxidative damage and also suggests possible antioxidant ability in animal fluids and tissues. (SANCHEZ-MORENO, LARRAURI & SAURA-CALIXTO, 1999). Several assays have been used to measure free radical scavenging capacity and the $DPPH^{\bullet}$ assay has been recommended by the International Organisation of Vine and Wine as a rapid and precise method for grape products. Studies have earlier examined the antioxidant properties of wine (DE BEER, et al., 2003; KATALINIC, et al., 2004; MUNOZ-ESPADA, et al., 2004). Antioxidant capacity of fruit juices is not always similar to those of fresh fruits and measurement in a wide range of food matrices raises discrepancies due to differences in plant cultivars (KARAKAYA, EL & TAS, 2001). Such inconsistencies reveal that certain properties of phenolic-rich products are influenced by polyphenolic composition, which is affected mainly by vintage, grape cultivar, production techniques and aging.

Electrospray ionization mass spectrometry (ESI-MS) with direct infusion of sample has appeared as a new alternative for the fingerprinting characterization of chemical mixtures, offering a fast and robust technique for typification of several beverages such as fruit juices (ROESLER, et al., 2007), yerba matte and green tea (BASTOS et al., 2007), whisky (MOLLER, CATHARINO & EBERLIN, 2005), wine (CATHARINO et al., 2006) and cachaça (DE SOUZA, et al., 2007). ESI-MS fingerprinting has therefore greatly

expanded the applicability of mass spectrometry to perform fast, selective and reliable characterization of products of different origins (MOLLER, CATHARINO & EBERLIN, 2007b). ESI-MS fingerprinting has also been proven a powerful technique for the typification and quality control of essential oils, fatty acids, organic acids and pigments in foods (CATHARINO, et al., 2005; MOLLER, et al., 2007a; MOLLER et al., 2007b). ESI-MS fingerprinting seemed to us to be also convenient for the direct analysis of grape juices, as most molecules bearing acidic or basic sites should be detected whereas MS/MS with collision-induced dissociation (CID) could be used for more detailed structural elucidation. This paper describes the characterization and quantitation of total phenolic contents and radical scavenging of concentrated grape juices during processing and storage and the first ESI-MS fingerprinting investigation of these juices.

2- Materials and methods

2.1 Samples and preparations

Concentrated grape juice samples of Concord and Isabel (known as "Isabella" in North-America) cultivars were received in February and March of 2006. Cultivars belong to *Vitis labrusca* species and are the most used for grape juice production in Brazil, with variations on their soluble solids contents: Concord between 14 to 16°Brix and Isabel between 15 to 19°Brix. Concentrated grape juices were provided by a national producer from Rio Grande do Sul - Brazil. Samples of both cultivars were also obtained at each step of the industrial process which consists of hot pressing of grapes and pasteurization of the must (80°C, 30 seconds) followed by filtration and concentration of juice to 68°Brix (98°C, 5 seconds). Concentrated juices were stored at 5°C in the dark simulating industrial storage conditions. Every 30 days two samples of each grape cultivar were placed under -18°C waiting for analysis, with maximum aging time of 10 months. Prior to analysis concentrated juices were reconstituted to 14°Brix by mixing 1mL juice to 3.85 mL of deionised water.

2.2 Determination of total phenols

Total phenols were measured by the Folin-Ciocalteu assay (SINGLETON & ROSSI JR, 1965) using gallic acid (Sigma-Aldrich, St Louis, MO, USA) for the standard curve and results expressed in mg gallic acid equivalents/L (GAE). Floating particles were removed by centrifugation and juice sample diluted 1:100 with deionized water, followed by colorimetric reading (in duplicate) at 760 nm with a Beckman spectrometer.

2.3 Determination of radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazil) (Sigma-Aldrich, Steinheim, BW, Germany) assay was used based on the methods of (BRANDWILLIAMS, CUVELIER & BERSET, 1995), modified by (KIM, et al., 2002). The absorbance was measured with a Beckman spectrometer at 517 nm before addition of samples and after 30 minutes; the difference was plotted on a Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (Sigma-Aldrich, St Louis, MO, USA) standard curve. Analyses were carried out in duplicates and the results expressed in mM Trolox equivalent (mM TE).

2.4 Electrospray ionization mass spectrometry fingerprinting

Grape juices were diluted in a solution containing 50% (v/v) chromatographic grade methanol (Tedia, Fairfield, OH, USA) and 50% (v/v) deionized water and 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). ESI–MS fingerprints in the negative ion mode of juices were acquired and accumulated over 60 sec and spectra were scanned in the range between *m/z* 250 to 600 to investigate processing and in the range of *m/z* 250 to 900 for cultivar comparisons, using a Micromass-Waters Q-TOF mass spectrometer (Manchester, England). Capillary and cone voltages were set to -3000 V and -40 V, respectively, with a de-solvation temperature of 100 °C. ESI-MS was preformed by direct infusion with typical flow rate of 10 µl min^{-1} using a syringe pump (Harvard Apparatus). Structural analysis of selected ions from the grape juices was performed by ESI-MS/MS. The ion of interest was selected and submitted to 15-45 eV collisions with argon in the collision hexapole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation.

2.5 Statistical analysis

The *t* test was applied to compare total phenols and radical scavenging activity averages between cultivars. To verify the relationships between parameters, Pearson correlation coefficients were calculated. Data analyses were conducted using Excel 97 (MICROSOFT CORPORATION, Washington, USA).

3- Results and discussion

Figure 1 shows radical scavenging activity (RSA) and total phenol (TP) contents during processing of concentrated grape juice.

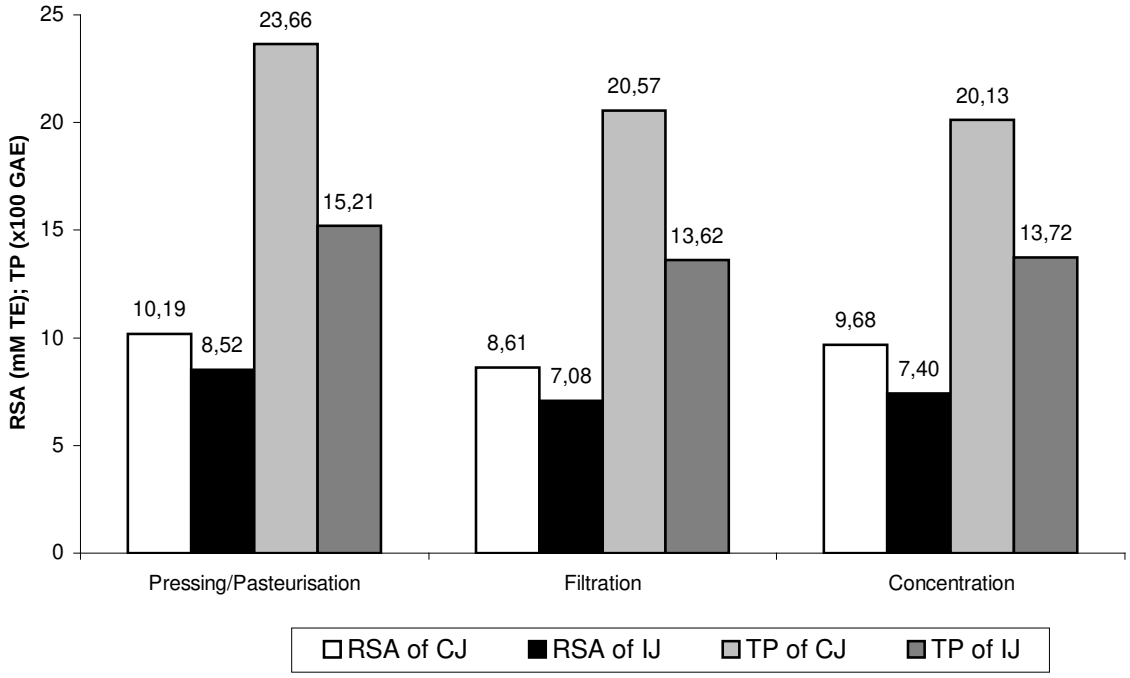

Figure 1. Radical scavenging activity (RSA) and total phenol contents (TP) during processing of Concord (CJ) and Isabel (IJ) grape juices. Variation between duplicates was less than 5%.

Concord juice (CJ) and Isabel juice (IJ) demonstrated diverse amounts of TP and RSA from the first step. A stable behaviour throughout the process was observed for both parameters and juices with some variation after heat treatment (pasteurization). Although

CJ showed *ca* 50% higher contents of TP, its RSA was in average 25% superior. Such disparities could be attributed to different phenolic composition, which would yield radical scavenging activity. Yildirim and co-workers (YILDIRIM, et al., 2005) have reported variations in total phenols and antioxidant activity during the steps of wine making (grape, pomace, juice, must and wine).

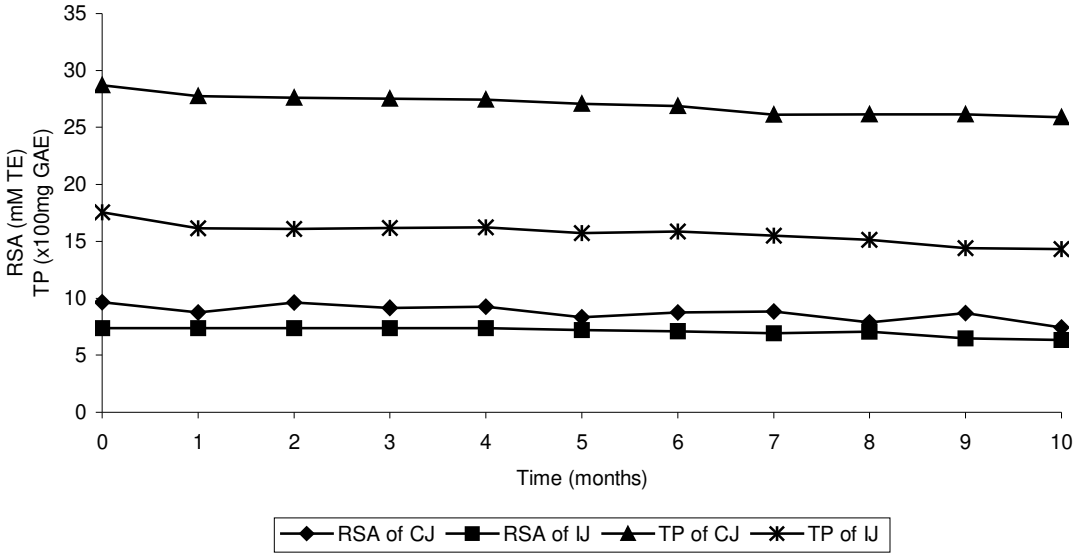

Figure 2. Radical scavenging activity (RSA) and total phenols content (TP) of Concord (CJ) and Isabel (IJ) grape juices stored at 68° Brix and 5° C. Variations between duplicates were less than 5%.

TP contents and RSA of CJ and IJ displayed a fairly stable behaviour during aging (Figure 2). TP contents varied from 2872.9 to 2587.6 GAE in CJ and from 1756.8 to 1428.9 GAE in IJ. RSA went from 9.68 to 7.45 mM TE in CJ and from 7.40 to 6.33 mM TE in IJ. Concord grape juice presented higher TP contents and RSA in the average of 10 months ($p<0.001$) and a positive and significant correlation was found between TP and RSA for Concord juice ($r=0.78$, $p=0.005$) and Isabel juice ($r=0.88$, $p<0.001$). TP retention percentage was 90% and 81% for CJ and IJ, respectively, whereas RSA retention was 77% and 86% for CJ and IJ, respectively. IJ portrayed higher RSA retention in spite of the higher TP loss. PÉREZ-VICENTE et al., 2004 observed similar behavior regarding TP

contents of pomegranate juice: a 2% loss during process and *ca* 20% decrease after 5 months aging. Contrary to our findings, however, no correlation was observed between TP and RSA, which increased by 10% after heat treatment and by 30% after the storage period. To investigate the contribution of a "phenolic unit" to the RSA of both juices, we calculated the mean ratio TP:RSA and found a value of 0.0045 for IJ and 0.0032 for CJ. These ratios indicate therefore that specific phenolic compounds or synergy among them were relevant for the greater radical scavenging power "per unit" of IJ.

Figure 3 shows ESI(-)-MS fingerprints of the Concord grape juices in different stages of processing and storage, which reveal significant and interesting composition changes. Given that Isabel juice demonstrated equivalent behavior, ESI-MS fingerprints of this cultivar are not shown. Note that the spectra of Figure 3 display characteristic profiles of mainly polyphenols and eventually hexose. The ESI(-)-MS fingerprints of juice samples show characteristic distributions of mainly the following compounds: dimethoxy-flavylium (DF), malvidin (M), dimmer of the hexose (H) and piceatanol-O-glucoside (PG) detected as the deprotonated molecules of *m/z* 313, 329, 359 and 405, respectively. Prior to heat treatment (Figure 3A), characteristic compounds were detected such as peonidin (P), caffeoyltartaric acid (CA) and peonidin-3-O-glucoside (P3G) identified as the marker ions of *m/z* 299, 311 and 461, respectively. The juice composition as identified by ESI-MS changes significantly after pasteurization (Figure 3B), being characterized by the predominance of three significant marker ions of *m/z* 313 (DF), *m/z* 329 (M) and *m/z* 359 (H), in a ratio of ca. 4:2:5. The ESI-MS fingerprint of the juice after concentration (Figure 3C) also changes with the clear detection of new major polar components (note those detected by the anions of *m/z* 293, 457 for instance), being characterized by the predominance of five significant major ions of *m/z* 293 (unknown), *m/z* 313 (DF), *m/z* 329 (M), *m/z* 359 (H) and *m/z* 405 (PG), in a ratio of ca 4:10:5:6:3. Figure 3D shows that after 8-month storage in concentrated and refrigerated conditions, the ESI-MS fingerprint of grape juice changes slightly being characterized now by two major marker ions of *m/z* 313 (DF) and *m/z* 359 (H) in a ratio of ca 9:10. Earlier, we (CATHARINO et al., 2006) had identified the ions of *m/z* 313 (DF), *m/z* 329 (M), *m/z* 359 (H) as diagnostic ions for the must of six varieties of grapes. The present results reveal that peonidin and peonidin-3-O-glucoside were replaced by malvidin and dimethoxy-flavylium as significant components

during grape juice processing and that malvidin and piceatanol-O-glucoside underwent reduction in the 8-month storage. These findings confirm that glucoside and highly hydroxilated anthocynins are less stable under oxidative and thermal conditions than their methylated forms (TALCOTT & LEE, 2002). In bottled wines (MUNOZ-ESPADA et al., 2004), have observed the presence of several anthocyanin aglycons in higher amounts than glucosides, depending on the grape cultivar.

The ESI-MS fingerprints of Figure 4 show similarities and some important differences between Isabel and Concord cultivars (after concentration). IJ (Figure 4A) is characterized mainly by three abundant ions of *m/z m/z* 313 (DF), *m/z* 329 (M), *m/z* 359 (H) in a ratio of ca. 9:3:10. CJ (Figure 4B) also produces a quite characteristic ESI-MS showing the predominance of five ions of *m/z* 293 (unknown), *m/z* 313 (DF), *m/z* 329 (M), *m/z* 359 (H), and *m/z* 405 (PG) in a ratio of ca 4:10:5:6:3. Concord juice depicted higher proportion of malvidin and lower of hexose dimmers than Isabel. These results are in agreement to the intense colour of CJ and higher sweetness of IJ.

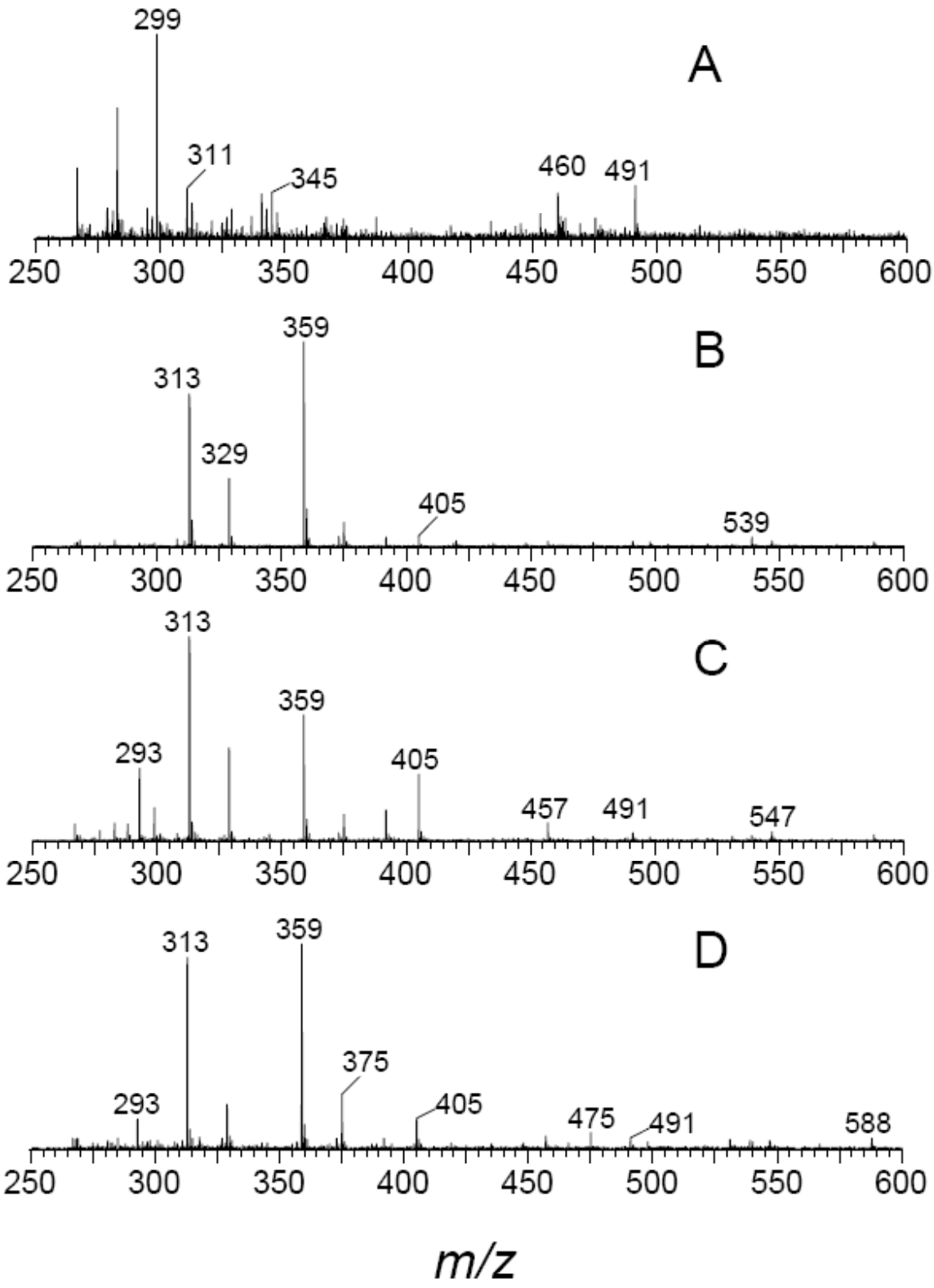

Figure 3: ESI(-)-MS fingerprints of Concord grape juice: (A) at pressing; (B) after pasteurization and filtration; (C) after concentration; and (D) after 8-month storage.

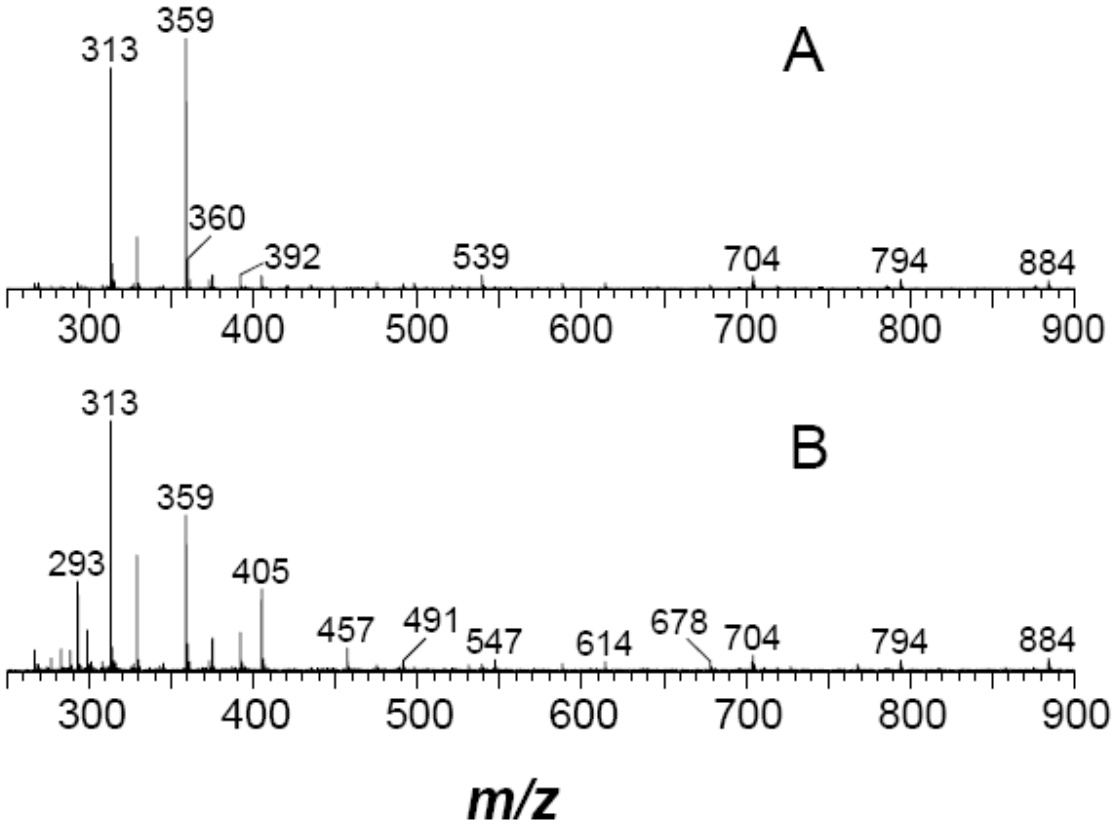

Figure 4: ESI-MS fingerprints of (A) Isabel and (B) Concord grape juices after concentration.

The investigated grape juices revealed similar TP and RSA values as reported for red wine (2036 GAE and 6-12 TE, respectively) and green tea infusion (1029 GAE and 8 TE, respectively), according to (GIL, et al., 2000). Phenolic composition of Isabel grape juice depicted a contribution to antioxidant activity, which indicates that particular alterations during processing/storage and specific compositions should be considered when assessing the intake of natural antioxidants and the oxidative damage of fruit beverages. The ESI-MS technique with direct infusion provided information of the mixture composition, particularly the changes in profile of major bioactive components. Concentrated and refrigerated storage of grape juices demonstrated to be effective to preserve juice quality in respect to phenolic compounds and oxidative status.

ACKNOWLEDGEMENTS

The authors thank finantial support from the Sao Paulo State Foundation (FAPESP) and the National Council for Scientific and Technological Development (CNPq), linked to the Brazilian Ministry of Science and Technology (MCT) for the postgraduate scholarship (process n° 131016/2006-7).

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CAPÍTULO 3:

Storage of Concentrated and ready-to-drink Grape Juices and impact on (+)-catechin and (-)-epicatechin.

(Artigo aceito para publicação no International Journal of Food Science and Technology).

Storage of concentrated and ready-to-drink grape juices and impact on (+)-catechin and (-)-epicatechin

Abstract:

Commercial concentrated Concord (CCJ) and Isabel (CIJ) grapes juices were stored under refrigeration while pasteurized ready-to-drink juices of the same grape cultivars (PCJ and PIJ) were kept at room temperature under indirect light for 10 months, simulating industrial storage conditions. (+)-catechin losses during storage ranged between 37% (PCJ) and 48% (PIJ); (-)-epicatechin recorded losses of 68% (CCJ) and 85% (CIJ). Total phenols reduction ranged from 7% (CCJ) to 16% (PCJ) and radical scavenging activity from 13% (PIJ) to 15% (CCJ and PCJ). Concentrated juices showed notably higher catechin amounts while Concord cultivar depicted greater phenolic contents. Despite the marked differences in phenolic and catechins contents in juices, not a correspondent disparity was encountered in the scavenging activity. CCJ yielded the highest radical scavenging activity during storage per phenolic unit. Process and storage impacted more fiercely catechins rather than total phenolics and radical scavenging activity during 10-month aging.

1- Introduction:

Epidemiologic studies have revealed that phenolic-rich diets significantly reduce incidence and mortality by degenerative diseases caused by oxidative stress. The main dietary sources are fruits, and plant-derived beverages such as fruit juices, tea, coffee and red wine (SCALBERT, JOHNSON & SALTMARSH, 2005). Among the various classes of phenolic compounds, flavan-3-ols exert certain physiologic properties, which may be the source of alleged health benefits derived from wine consumption, according to GÜRBÜZ et al., (2007). Dietary intervention studies supports flavan-3-ol-rich foods and beverages as beneficial to the vascular function and platetel reactivity, thus promoting cardioprotective effects (KEEN et al., 2005). In grape juices, flavan-3-ols are found most abundantly in the monomeric forms of catechins [(+)-catechin and (-)-epicatechin] with large differences among cultivars (JAROWISKY & LEE, 1987; LEE & JAROWISKY, 1987; SPANOS & WROLSTAD, 1990; AUW et al., 1999).

Catechins are among the three most well-absorbed polyphenols by humans, after gallic acid and isoflavones, which draws particular focus towards them (MANACH et al., 2005). In human intervention studies catechins has been associated with increased plasma antioxidant activity, increased plasma ascorbate concentrations, increased resistance to LDL oxidation and decreased plasma lipid peroxide and malondialdehyde concentrations (LOTITO & FRAGA, 1997; KAMPA, et al., 2000; KIMURA, et al., 2002; WILLIAMSON & MANACH, 2005). In an epidemiological study, ARTS et al. (2001a,b) demonstrated a positive association between catechin consumption and reduced mortality by chronic diseases. The principal presumed property of polyphenols in general and catechins in particular is the antioxidant capacity, which is relevant for both food manufacturers and health professionals. In fact, apart from the recognized *in vivo* antioxidant activity, it has been reported the use of catechins as inhibitors of cholesterol oxidation in meat products (OSADA, et al., 2001).

According to CHEYNIER (2005), polyphenolic compounds are highly unstable, reacting among themselves resulting in various products during food process and storage. In wines, catechins among other phenolics, undergo important changes during aging resulting in known and unknown phenolic species. Such modifications are overlooked in

most studies concerning food composition. In 2000, ARTS, PUTTE & HOLLMAN affirmed that epidemiological research required reliable data on catechins on foods, meaning that studies on changes during process or aging of products should be carried out to provide extra data on the subject.

Although some studies have already been published regarding the modifications in phenolic contents during storage, the design of present work highlights catechins contents and contemplates sampling of commercial products and real aging time and conditions. Furthermore, measurement of the radical scavenging activity throughout the storage period may support predict changes in the antioxidant capacity. Such assumptions justify the objectives of this work, which are to verify the impact of storage conditions on commercial grape juices in relation to total phenols, catechins monomers and antioxidant activity.

2- Materials and methods

2.1 Samples and preparations

Concentrated and ready-to-drink grape juices of Concord and Isabel ("Isabella", the original North-American name) cultivars were received in February and March of 2006. Cultivars belong to *Vitis labrusca* species and were chosen for the experiment given their frequent use for juice process. Concentrated juices were supplied by a large manufacturer after process which consists of pressing with simultaneous pasteurization followed by concentration to 68°Brix. Upon arrival, concentrated juices were stored in 250mL plastic recipients under refrigeration at 5°C in the dark. Ready-to-drink pasteurized grape juices (soluble solids ranging from 14°C and 16°C Brix) were obtained from a small private manufacturer, after pressing of grapes followed by pasteurization and immediate bottling. Juices were stored in their commercial transparent green glass bottles under indirect light and room temperature (20-25°C). Both storage situations simulated real conditions at each industry/warehouse. Every 30 days two samples of each grape cultivar were taken from the specific storage condition and placed at a freezer at -18°C in the dark waiting for analysis, with maximum aging time of 10 months. Prior to analysis concentrated juices were reconstituted to 14°Brix by mixing 1mL juice to 3.85 mL of deionised water.

2.2 Determination of total phenols

Total phenols were measured by the Folin-Ciocalteu assay (SINGLETON & ROSSI, 1965) using gallic acid (Sigma-Aldrich) for the calibration of standard curve and to express the results in mg gallic acid equivalents/L (GAE). Sample dilution brought concentrated juice to 14° Brix as described in item 2.1 and floating particles were removed by centrifugation at 3,000 rpm for 15 minutes; the supernatant was then diluted 1:100 with deionized water. Colorimetric results of duplicates were read at 760 nm with a Beckman spectrometer and plotted in the standard curve (0.01 – 0.05 mg/mL)

2.3 Determination of radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazil) (Sigma) was used to evaluate free radical scavenging activity of juices using the methods according to Brand-Williams, Cuvelier & Berset (1995), modified by Kim et al. (2002). In the radical form, $DPPH^{\bullet}$ presents a maximum absorption at 517 nm, but upon reduction by a radical scavenger, a pale-yellow non-radical form is produced. Methanolic solutions of DPPH (100 μm) were prepared daily using methanol 80% in water. Samples aliquots of 0.1 mL were added to 3.9 mL fresh DPPH methanolic solution and the mixtures were kept in the dark for 30 minutes at room temperature (25ºC). The absorbance was measured with a Beckman spectrometer before addition of samples and after 30 minutes in the presence of samples. A standard curve of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (Sigma-Aldrich) was built in concentrations of 0,08-1,28 mM/L in methanol 80%. Analyses were carried out in duplicates and the results expressed in mM Trolox equivalent (mM TE).

2.4 Determination of total catechins [(+) catechin and (-) epicatechin]

The two monomers were analysed by HPLC with fluorescent detection. The method of ARTS, PUTTE & HOLLMAN (1998) for an optimised determination of catechins in fruits was used with an adaptation for fruit juices described by ARTS & HOLLMAN (2000). Each concentrated juice sample was reconstituted to 14° Brix and when needed for better plotting on the standard curve, reconstituted or commercial juices were diluted again 1:2 with deionised water. Samples were then filtered through a 0,45μm Millex HV (PVDF)

disposable syringe filter, 33mm diameter. The standards of (+)-catechin (purity > 90%) and (-)-epicatechin (purity ≥ 96%) were obtained from Sigma Aldrich (São Paulo, SP). The stock solutions contained 0,2 mg catechins/mL methanol and were stored between below 4ºC. The standard curves were obtained using concentrations of 2, 4, 6, 8, 10 and 12 mg/L. A Perkin Elmer High Performance Liquid Chromatograph (HPLC) equipped with a binary pump and manual sampler was used. The column was a (250 mm x 4.6 mm) GL Science, Inertisil ODS - 3,5 μm and the flow rate of the solvent was 1 mL/min. The mobile phase consisted of 5% acetonitrile (solvent A) and 25% acetonitrile (solvent B) in 0.025M phosphate buffer, pH 2.4 with the following gradient: 0-15 min, 45% B; 15-28 min, linear gradient from 45 to 70% B; 28-30 min, linear gradient from 70 to 45% B. The volume injected was 10 μl and detection was by fluorescence (wavelength of 280 nm excitation and 310 nm emission). The retention time of (+)-catechin varied from 11 to 15 minutes and that of (-)-epicatechin from 18 to 22 minutes. Analyses were conducted in duplicates. To determine the detection limit, ten noise concentrations were taken at random and the standard deviation multiplied by three. Quantification limit was determined by injecting standard solutions and verifying the minimum concentration that provided accurate integration of peak. In this study, detection limit was 0.05 mg/L and quantification limits were 1.00 mg/L for (+)-catechin and 2.00 mg/L for (-)-epicatechin.

2.5 Statistical analysis

Averages of duplicates as well as standard deviations and variation coefficients were calculated to evaluate precision of determinations. In the present study, variation coefficients were lower than 5% for total phenols and radical scavenging activity and lower than 10% of (+)-catechin and (-)-epicatechin determinations. The t-test was applied to compare averages of 10-month measurements of parameters; to investigate relationships between them, Pearson correlation coefficients were used.

3. Results and discussion

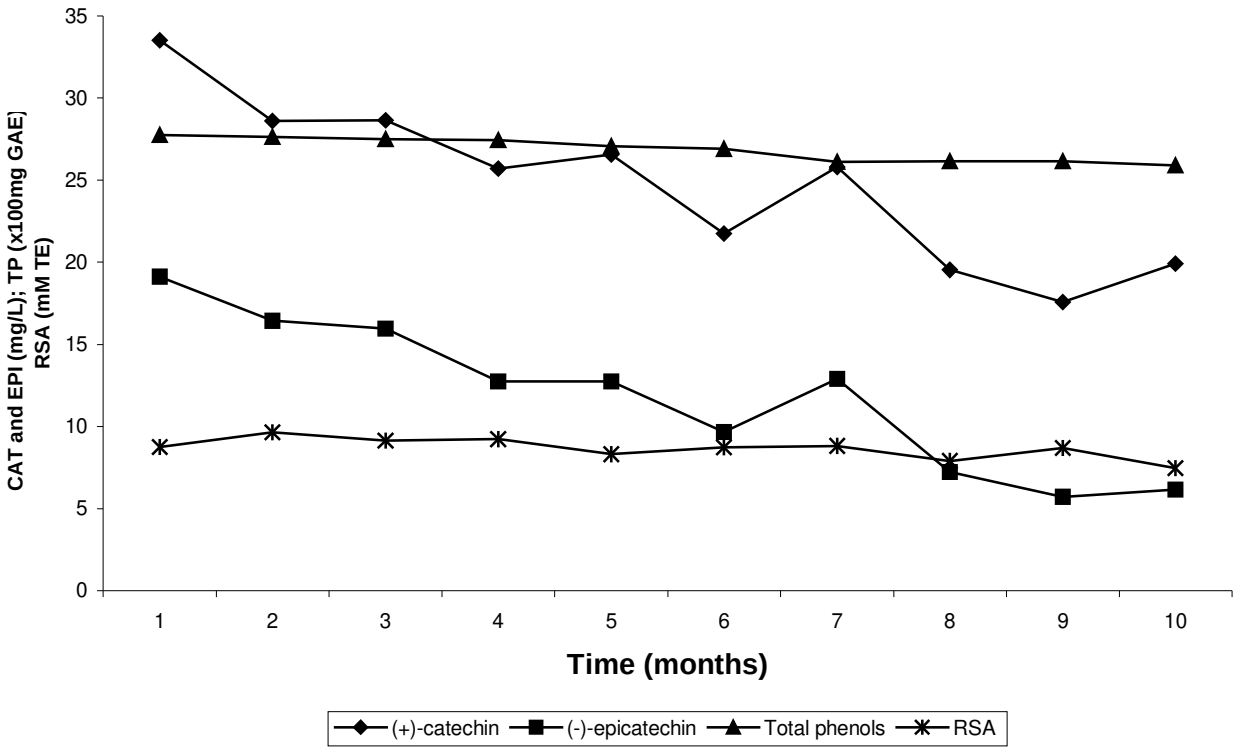

Figure 1. (+) catechin (CAT), (-)-epicatechin (EPI) total phenols (TP) and radical scavenging activity (RSA) of concentrated Concord grape juice (CCJ) during 10 months storage under refrigeration. Variation coefficients were lower than 5% for TP and RSA and lower than 10% of CAT and EPI.

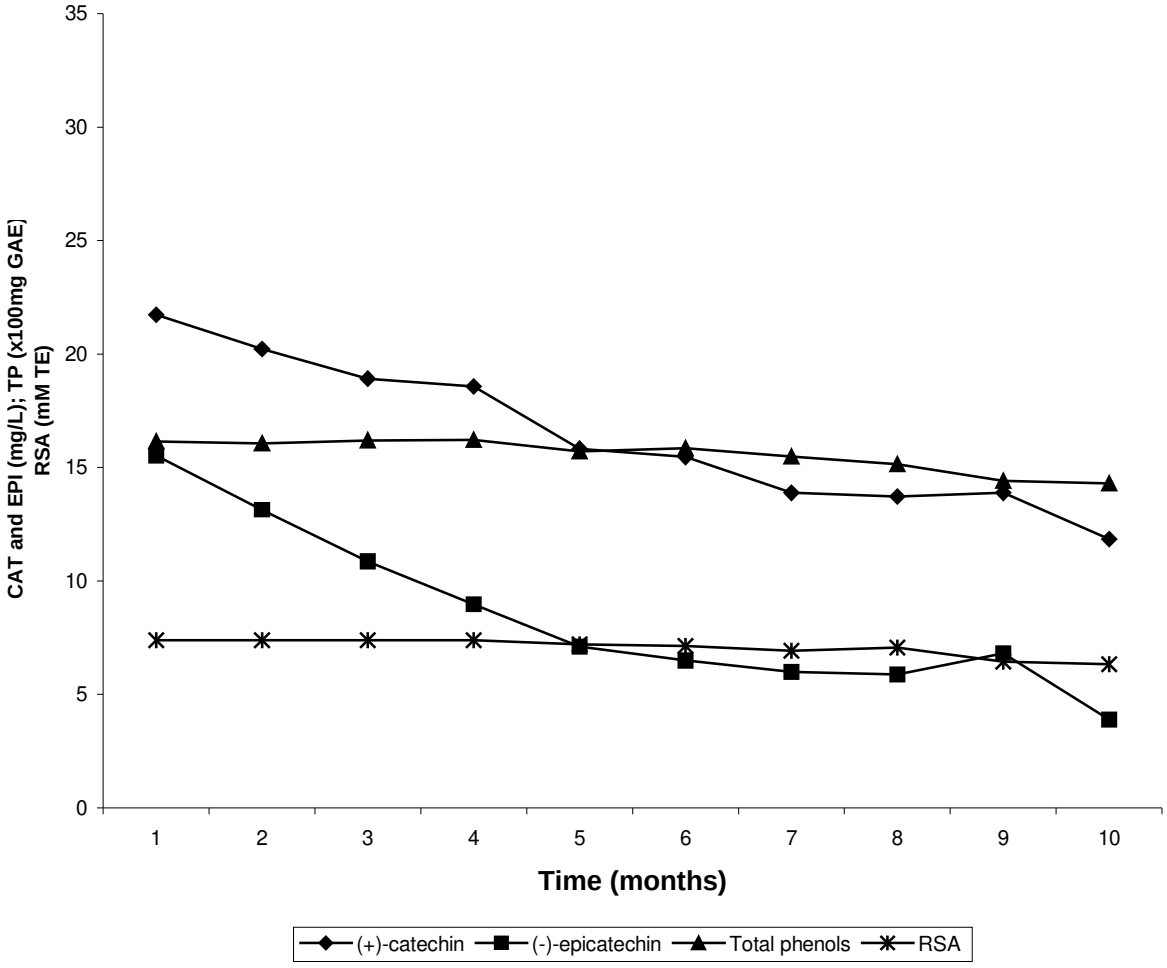

Figure 2. (+) catechin (CAT), (-)-epicatechin (EPI) total phenols (TP) and radical scavenging activity (RSA) of concentrated Isabel grape juice (CIJ) during 10 months storage under refrigeration. Variation coefficients were lower than 5% for TP and RSA and lower than 10% of CAT and EPI.

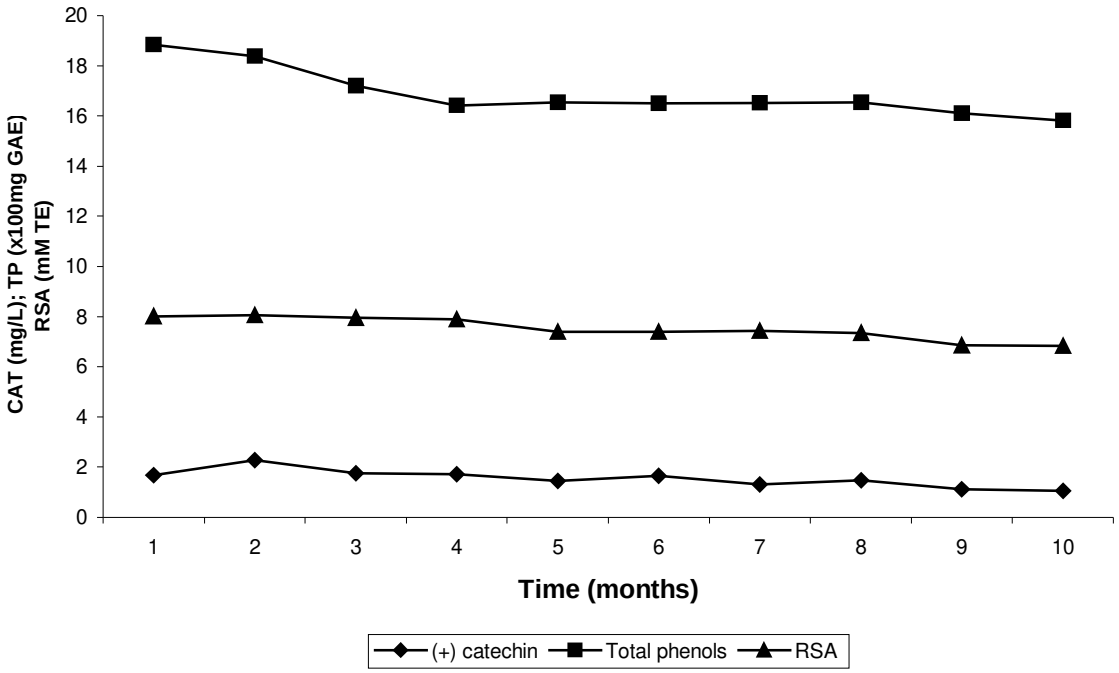

Figure 3. (+) catechin (CAT), (-)-epicatechin (EPI) total phenols (TP) and radical scavenging activity (RSA) of pasteurized Concord grape juice (PCJ) during 10 months storage in transparent green glass bottles under indirect light and at room temperature. Note: (-) epicatechin concentrations were below quantification limits for the substance (2.00 mg/L). Variation coefficients were lower than 5% for TP and RSA and lower than 10% of CAT and EPI.

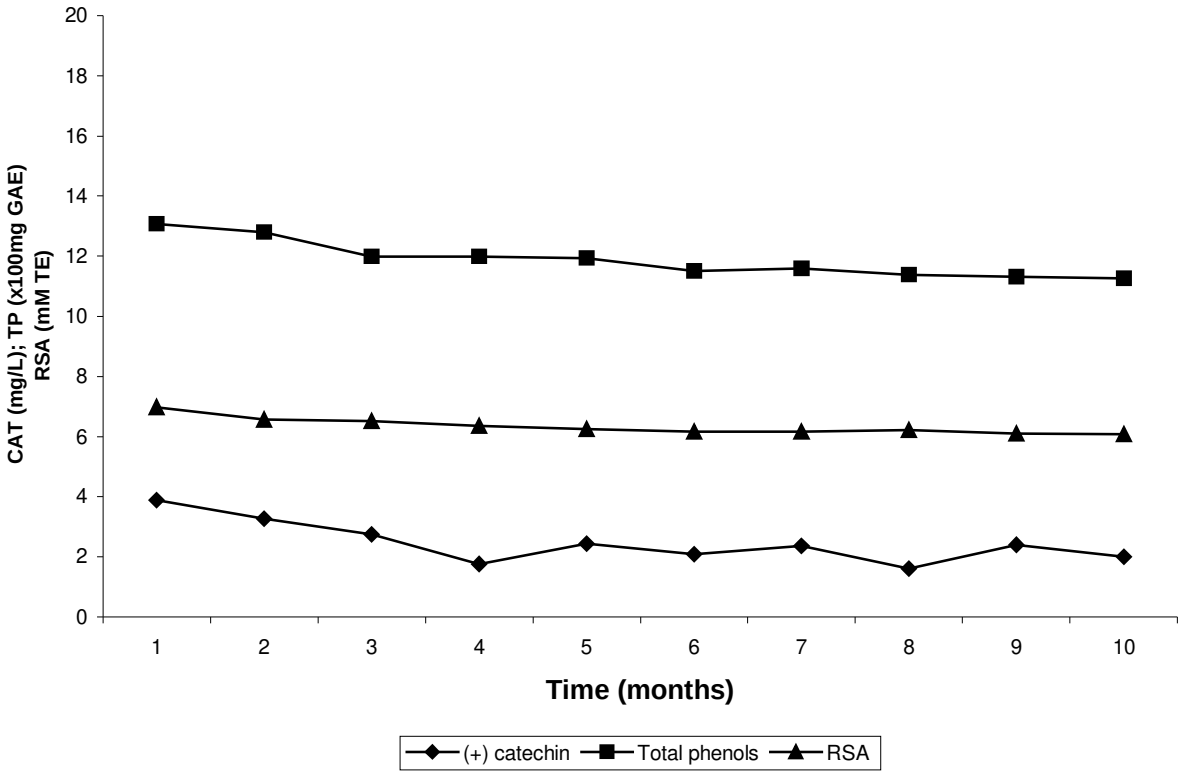

Figure 4. (+) catechin (CAT), (-)-epicatechin (EPI) total phenols (TP) and radical scavenging activity (RSA) of pasteurized Isabel grape juice (PIJ) during 10 months storage in green glass bottles under indirect light and at room temperature. Note: (-) epicatechin concentrations were below quantification limits for the substance (2.00 mg/L). Variation coefficients were lower than 5% for TP and RSA and lower than 10% of CAT and EPI.

Figure 1 shows diverse behaviours of analysed parameters during the 10-month storage of concentrated Concord grape juice (CCJ) under refrigeration. CAT and EPI depicted reduction from 33.51 to 19.93 mg/L and 19.12 to 6.15 mg/L respectively. TP and RSA showed a more moderate decrease: from 2775.0 to 2587.6 mg GAE and from 8.77 to 7.45 mM TE respectively. In terms of retention during storage, CAT and EPI demonstrated important losses (41% and 68% respectively), which did not correspond to same degree of reduction in TP or RSA (7% and 15%, respectively). The results for concentrated Isabel grape juice (CIJ) (figure 2) under the same storage conditions showed similar performance,

in spite of the different absolute values for most parameters. CAT and EPI depicted reduction from 21.73 to 11.85 mg/L and 25.54 to 3.88 mg/L, respectively (45% and 85% loss, respectively). TP and RSA varied from 1615.0 to 1429.0 mg GAE and from 7.38 to 6.33 mM TE, respectively (12% and 14% loss respectively).

Figure 3 displays the development of analysed parameters of pasteurised Concord juice (PCJ) during storage under indirect light and room temperature. CAT depicted reduction from 1.68 to 1.06 mg/L (37% loss) and EPI concentrations were below quantification limit for the substance (2.00 mg/L). TP and RSA showed reduction from 1884.3 to 1582.3 mg GAE and from 8.02 to 6.83 mM TE (16% and 15% loss, respectively). Pasteurized Isabel juice (PIJ), under similar storage settings demonstrated similar modifications (figure 4). CAT depicted reduction from 3.89 to 2.01 mg/L (48% loss) and EPI concentrations were below quantification limit. TP and RSA decreased from 1306.9 to 1126.9 mg GAE and from 6.98 to 6.08 mM TE (14% and 13% loss, respectively).

Considering the four investigated juices, CAT losses ranged between 37 and 48% and EPI (detected only in concentrated juices) of 68% and 85%, demonstrating an analogous pattern during aging, regardless of cultivar and storage differences. EPI depicted greater reduction than its isomer CAT, probably due to higher reactivity. These observations agree with the results of FREITAS, GLORIES & LAGUERRE (1998) using a model assay to study the oxidative decomposition of total catechins extracts during a 60-day period. The authors observed important decrease of both isomers with (-)-epicatechin being more oxidizable than (+)-catechin. In our study, TP and RSA values of the four juices did not decrease at the same rate as flavan-3-ols: TP losses ranged from 7% to 16% and RSA from 13% to 15%, signaling that other phenolic compounds, rather than monomeric catechins contributed to the radical scavenging capacity. Moreover, despite the different storage conditions, RSA retentions were quite similar in all juices. In teas, LEUNG et al. (2001) had similar findings, demonstrating that teaflavins, originated from oxidized and dimerized catechins possessed the same radical scavenging activity as the initial monomers. TALCOTT & LEE (2002) tested antoxidant activity (using ORAC assay) of Muscadine grape juices during a 60-day storage and found that process methods rather than storage conditions were important for retention of radical scavenging properties, which partially

agrees with our investigations. Actually, neither storage nor process impacted RSA, which was more related to cultivar with Concord juices showing higher activity. In dessert prepared with concentrates of grape, cherry, raspberry, blackberry and backcurrant, GARCÍA-ALONSO et al. (2003) verified that although storage at different temperatures impaired especific phenolics compounds, antioxidant activity demonstrated modest change after 12 months. Some authors attributed the loss of phenolic compounds during aging to enzymatic and non-enzymatic reactions (GASPAR, et al., 1998; ES-SAF, CHEYNIER & MOUTOUNET, 2003).

When absolute averages of the 10-month period were compared, statistical differences among juices were detected. CCJ contained the highest values of CAT, TP and RSA, followed by CIJ ($p<0.001$) (no difference for EPI); PCJ showed higher RSA than CIJ. PCJ depicted higher amounts of TP and RSA than PIJ, but lower CAT. In absolute terms, concentrated juices showed notably higher catechin contents, probably due to the process itself which consists of hot pressing (with simultaneous pasteurization) followed by concentration at 98°C for 5 seconds. In contrast, pasteurised juices, which process consists of cold pressing showed practically 10% of the catechin amounts found in concentrated juices. It has earlier been described by MUSINGO et al. (2001) and FULEKI & RICARDO-DA-SILVA (2003) that flavan-3-ols concentration suffered influence of processing methods and that hot pressing enhanced the extraction of the substances. Incidentally, the process seems also to alter the extraction of phenolics as a whole, as TP contents of Concord or Isabel juices were higher in concentrated rather than pasteurized juices. Likewise, at either process settings, Concord grape juices depicted higher values than Isabel grapes.

Aging was associated with decrease in CAT, EPI, TP and RSA in all four juices ($p<0.05$). Despite of the high reduction suffered by catechins, TP and RSA, depicted retention of over 84% during 10 months aging in both storage condition and cultivars. ZAFRILLA et al. (2003) observed similar event with wines stored for 7 months at 20°C in the dark: antioxidant activity measured by DPPH assay were not significantly different, although decrease in the concentration of some phenols was observed. Radical scavenging activity showed stronger positive correlation with TP rather than CAT or EPI for CCJ, CIJ and PIJ ($p<0.05$); for PCJ a stronger correlation with CAT at the same significant level was

detected. Similarly, FRANKEL, WATERHOUSE & TEISSENDRE (1995) tested phenolic compounds from wine on their capacity of inhibiting oxidation of human LDL and verified a stronger positive relation with total phenolics rather than (+)-catechin or (-)-epicatechin.

The present results reveal that despite the large differences in TP contents found in the grape juices, not a correspondent disparity was encountered in RSA. Hence, in order to verify the radical scavenging potential for each "phenolic unit", a RSA:TP rate (average values) was calculated and compared. In concentrated juices, the relation was 3.2 for CCJ and 4.5 for CIJ and in pasteurized juices, 4.4 and 5.3 for PCJ and PIJ, respectively (all relations $x10^{-3}$). The numbers exposed that although PIJ depicted the lowest TP concentration, it yielded the highest RSA:TP ratio, indicating that specific phenolic compounds and/or synergistic effects are relevant in the assessment of radical scavenging activity *in vitro*.

4. Conclusions

Process and storage impacted catechins contents in grape juices. Although concentrated juices depicted important reduction in total catechin levels, average amounts were significantly higher than in pasteurized beverages, with important differences in cultivars. That should be taken into account in future studies assessing catechins consumption of grape products. Storage conditions were not a relevant factor in the retention of total phenolics and radical scavenging activity *in vitro* during 10-month aging. Moreover, the results of this study revealed that notwithstanding the importance of the quantity of phenolics, the antioxidant power per phenolic unit should be addressed when evaluating antioxidant activity of phenolic-rich foodstuffs. This information may be useful for the design and interpretation of future studies.

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CAPÍTULO 4:

"Concord grape juice and Norbixin attenuates Acetaminophen-induced oxidative stress in rats".

(A ser submetido ao Archives of Biochemistry and Biophysics).

Concord grape juice and Norbixin attenuates Acetaminophen-induced oxidative stress in rats

Abstract

Rats Wistar male received orally twice a day 1 ml of Concord grape juice (CGJ) (polyphenolic concentration 24 mg/ mL.) or 1 mL of Norbixin (Nb) water solution (24 mg/ mL) during 30 days On the 29th and 30th day, one dose of acetaminophen (AAP) (100 mg/ kg body weight, intaperitonially) was administrated. Lipid peroxidation index (TBARS) and antioxidant enzymes (SOD, MnSOD, CuZnSOD, GPX, GPx Se-dependent, catalase) were measured in liver and kidney. AAP liver and kidney show significantly increase ($p \leq 0.05$) in TBARS levels. Liver presents an increase of 43% and kidney 16.3%. In the liver CGJ+AAP and Nb+AAP lowered significantly the peroxidation caused by AAP in 18.7% and 21.0% respectively. On the other hand, in the kidney CCJ+AAP and Nb+AAP reduces the peroxidation in 7.1% and 5.3% respectively and not differ significantly ($p \leq 0.05$) of AAP group. CGJ and Nb tested alone did not differ to Control group. The catalase activity in CGJ+AAP shows an increase of 200% in the liver and 100% in the kidney in comparison to AAP group ($p \leq 0.05$). Liver Nb+AAP catalase activity was increased in 54% to AAP group. Kidney did not show increases in catalase activity in comparison to AAP group. Liver and kidneys showed distinct responses in the antioxidants presence, but both CGJ and Nb reduces AAP-toxicity induced.

Keywords: Oxidative stress, Concord grape juice, Norbixin, Acetaminophen, Antioxidant enzymes, liver, kidney

1 Introduction

Many epidemiological studies already reported a positive correlation between diets rich in plant foods and less degenerative diseases (RIMM, 2002). Fruits, vegetables and beverages, such as wine, juices and tea contain substances as vitamins, organic acids, carotenoids and polyphenols that contribute to the health effects. These substances can serve as chemopreventive agents in disease states involving oxidative stress or attenuating the toxicity caused by certain drugs and chemicals (MANACH, et al., 2005).

Polyphenols have been described to exert a variety of biological actions such as free radical scavenging, metal chelation, modulation of enzymatic activity and more recently to affect signal transduction, activation of transcription factors and gene expression (NARDINI & GHISELLI, 2004). Concord grape juice is a rich source of polyphenolic compounds mainly catechins, epicatechins, quercetins, anthocyanins, and proanthocyanidins (RIBÉREAU-GAYON, & PEYNAUD, 1971; FULEKI & RICARDO-DA-SILVA, 2003) substances that show great potential antioxidant (RICE-EVANS, 2002). In humans studies, the consumption of Concord grape juice increased protection against low-density lipoprotein cholesterol (LDL) oxidation, decrease platelet aggregation and endothelial adhesion, to mediate nitric oxide production (O' BYRNE, 2002). In rats studies, grape juice polyphenols have been demonstrated capacity to suppress cancer cell growth, and to reduce oxidative stress (JUNG, 2006).

Other class of natural antioxidant as the carotenoids, are reported as chain breaking by scavenging and deactivating the free radicals both *in vitro* and *in vivo* (VANDENBERG, et al, 2000). The Bixa orellana L. seeds are a rich source of orange-red carotenoids pigments are known as annatto (E160b) by the food industry. Their components are Bixin ($C_{25}H_{30}O_4$), an oil-solube carotenoid and a dicarboxylic water-solube fraction called Norbixin ($C_{24}H_{28}O_4$). Industrially Norbixin is obtained by the hydrolytic removal of the methyl ester group from Bixin by saponification (KOVARY et al, 2001). Bixin has demonstrated antioxidant capacity acting as a quencher of $^1O_2$ and as a scavenger of $O^{\bullet}$, peroxynitritie and $OH^{\bullet}$ (DI MASCIO et al., 1990; ZHAO el at.,.1998). Previous research has demonstrated that Bixin / Norbixin been able to regulate levels of LDL and HDL in hyperlipidemics rabbits (SANTOS, et al., 2002). Other studies showed the capacity of

norbixin to induce the activity of cytochrome P450 monoxigenases system, responsible for the reactions of phase I during the hepatic xenobiotics metabolism (JEWELL & O'BRIEN, 1999; DE-OLIVEIRA, et al., 2003).

Acetaminophen (AAP), also known as Paracetamol, is widely used as analgesic and antipyretic drug (MIROCHNITCHENKO, 1999). At therapeutic dosis, under normal conditions, AAP is mainly metabolised by undergoing sulfitation and glucuronidation (BESSEMS & VERMEULEN, 2001). A small amount of drug goes through the cytocrome P450 and is metabolised into reactive intermediate N-acetyl-benzoquinoneimine (NAPQI), which is in turn detoxified by reaction with glutathione (GSH) (ALBANO, 1985). When in overdoses, AAP causes GSH depletion and the metabolite covalently binds to cellular macromolecules leading to tecidual injury (BIRGE, 1990; SALMINEN, 1998). Another theory states that NAPQI cause oxidative stress by depletion of cellular glutathione, a natural antioxidant, leaves the cell vulnerable to ROS following AAP administration (GIBSON, 1996). AAP is extensively used in vivo experimental models to induce oxidative damage (BESSEMS & VERMEULEN, 2001). Liver is the target organ for AAP toxicity, however, many organs such as renal, cardiac and central nervous systems are also attemppted by its antioxidation capacity (THOMAS, 1993).

The present study evaluated the antioxidant capacity of Concord grape juice (CGJ) and norbixin (Nb) against oxidative stress induced by Acetaminophen (AAP) administration.

2 Materials and Methods

2.1 Chemicals

Thiobarbituric acid and the reagents used to measure the antioxidant and the oxidative stress markers enzyme activities were purchased from Sigma Chemical Co (St Louis, USA) unless Acetaminophen (Johnson ®, Brazil). All the other reagents used were of analytical grade. All the solutions were prepared with ultra-pure water Milli-Q® system.

2.2 Grape juice and norbixin

The concentrate Concord grape juice (polyphenolic content 24 mg gallic acid equivalents/ ml) was provided by a national producer from Rio Grande do Sul - Brazil. Norbixin (Nb) (powder 95% purity, Rhodia, Paulínia, Brazil) were administrated in water solution (24 mg/ ml).

2.3 Animals

The experiment was carried out with the approval of the Institutional Committee for Ethics in Animal Research – UNICAMP (Protocol 1129-1) in compliance with Ethical Principles for Animal Research of the Brazilian College for Animal Experimentation. Male Wistar rats (70 – 80g) were obtained from the Central Laboratory Experimentation Animal of UNICAMP. Before any experience, all animals were kept one week under the same laboratory conditions under temperature (22 ± 2ºC), 12-h light/dark cycle, controlled humidity (50-60%) and air circulation. The rats had free access to standard rat diet (Purina Chow®, Brazil) and water.

2.4 Experimental design

After acclimation period, rats were randomly divided into six groups of 6 animals each. Group I: normal control; Group II: normal + Concord grape juice (CGJ); Group III: normal + norbixin (Nb); Group IV: Acetaminophen control (AAP); Group V: Concord grape juice + Acetaminophen (CGJ+AAP); Group VI: Norbixin + Acetaminophen (Nb+AAP). During 30 days, the groups received orally by *gavage* the following administrations: Groups I and IV: 1 mL of water (twice/ day); Group II and V: 1 ml of CGJ (twice/ day); Groups III and VI: 1 mL of Nb water solution (twice/ day). Animals of groups IV, V e VI received on the 29th and 30th day, one dose of acetaminophen (100 mg/ kg body weight, intaperitoneally). After 24 h the rats were anaesthetized with pentobarbitone (50 mg/ kg i.p); the blood was obtained by cardiac puncture until death. Immediately, liver and kidney were excised, perfused in ice-cold isotonic saline; one part of organ was collected to histological analysis and another one was quickly frozen and stored in nitrogen liquid for posterior analysis.

2.5 Assay for lipid peroxidation

Tissue lipid peroxide levels were determined as Thiobarbituric Acid Reactive Substances (TBARS) by the method of BUEGE & AUST, 1978 modified by HERMES-LIMA & STOREY, 1995. Frozen samples were homogenized (1:20 w/v) in ice-cold 1.1% phosphoric acid. Each 0.4 ml of those homogenates were mixed with 0.4 ml of 1% w/v thiobarbituric acid, 50 mmol l^{-1} NaOH, 0.1 mmol l^{-1} butylated hydroxytoluene solution and 0.2 ml of 7% phosphoric acid (all the solutions were kept on ice during manipulation to avoid side reactions). Subsequently, samples (at approx. pH 1.5) were heated for 15 min to 98° C and then 1.5 ml of butanol were added in each one tubes. Finally, the tubes were vigorously vortexed and centrifuged for 5 min at 2000 *g*. The organic layers were removed and placed in quartzo cuvettes. The absorbances were measured spectrophotometrically at 532 nm. The thiobarbituric acid solution was replaced by 3 mmol l^{-1} HCl for the blanks. An extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} was used to express final TBARS values.

2.6 Antioxidant enzymes activities

Total superoxide dismutase (SOD) activity was determined from the rate of inhibition of the oxidation of ferricytochrome-C at 550 nm in a standard reaction medium (McCORD & FRIDOVICH, 1969). The MnSOD activity was measured after inhibition of the Cu/Zn isoenzyme by addition of 1 mM KCN (BEAUCHAMP & FRIDOVICH, 1973). Catalase activity was determined by measuring the decrease in absorption of H_2O_2 at 240 nm (NELSON & KIESOW, 1972). Glutatione peroxidase (GPx) was determined by measuring the $NADPH+H^+$ oxidation rate in the presence of GSH and GSH redutase (LAWRENCE & BURK, 1976).

2.7 Protein Concentration

The protein concentration was determined by the method of LOWRY et al. (1951) using bovine albumin as a standard.

2.8 Histological analysis

For histological analysis liver and kidney samples were divided and both prepared to LM and TEM study. Paraffin sections hematoxilin-eosin stained, were analysed and the images registered; diaminobenzidine (DAB) reactions were carried in order to evaluate peroxisomes status. Epon sample inclusions and respective sections were prepared to TEM study in a Zeiss CEM 902 microscopy model.

2.9 Statistical Analysis

Results are expressed as mean ± standard deviation. Data were analysed by ANOVA followed by LSD Fisher and Dunnet, and Statistical significance were considered when $p \leq 0.05$. For all biochemical analysis was using the software XLSTAT® 2007.7 for Windows®.

3. Results

3.1 Lipid Peroxidation

AAP- Liver and kidney show significantly increase ($p \leq 0.05$) in TBARS levels (tables 1 -2), demonstrating that AAP induces lipid peroxidation. Liver presents an increase of 43% and kidney 16.3%. In liver CGJ+AAP and Nb+AAP lowed significantly the peroxidation caused by AAP in 18.7% and 21.0% respectively. In the kidney CGJ+AAP and Nb+AAP the peroxidation was reduced in 7.1% and 5.3% respectively, these values did not differ significantly ($p \leq 0.05$) of AAP group. Finally, CGJ and Nb tested alone did not differ to control group.

Table 1- Effect of Concord grape juice and Norbixin on TBARS and antioxidant enzymes activities in liver

Parameters	Control	AAP	GGJ+AAP	Nb+AAP	CCJ	Nb
			Liver			
TBARS nmol/ mg protein	0.30 ± 0.02	0.43 ± 0.07[a]	0.35 ± 0.03[b]	0.34 ± 0.01[b]	0.34 ± 0.03[b]	0.33 ± 0.02[b]
Total SOD U/ mg protein	25.67 ± 0.87	37.51 ± 2.10[a]	37.51 ± 1.44[a]	39.87 ± 1.26[ab]	53.58± 0.81[ab]	45.24± 0.56[ab]
MnSOD U/ mg protein	11.20 ± 0.57	9.84 ± 0.56[a]	10.81 ± 0.47	13.68 ± 0.79[ab]	13.32± 1.18[ab]	15.24± 0.68[ab]
CuZnSOD U/ mg protein	14.47 ± 1.35	27.67 ± 2.05[a]	26.70 ± 1.89[a]	26.20 ± 1.88[a]	40.26 ±1.72[ab]	30.00 ± 0.47[a]
Total GPx U/ mg protein	12.55 ± 0.67	16.38 ± 1.10[a]	9.64 ± 0.92[ab]	12.44 ± 1.10[b]	11.23 ± 0.70[b]	8.68 ± 0.59[ab]
GPx-se-dependente U/ mg protein	6.03 ± 0.33	6.01 ± 0.39	3.92 ± 0.93[ab]	2.64 ± 0.61[ab]	2.31 ± 0.21[ab]	2.80 ± 0.57[ab]
Catalase pmol/ mg protein	0.82 ± 0.04	0.87 ± 0.03	2.78 ± 0.08[ab]	1.34 ± 0.02[ab]	0.25 ± 0.03[ab]	0.35 ± 0.03[ab]

Results are mean ± S.D., n=5 AAP - acetaminophen; CCJ - Concord grape juice; Nb - Norbixina.
[a] Significantly different from Control, p≤ 0.05. [b] Significantly different from AAP, p≤ 0.05

Table 2- Effect of Concord grape juice and Norbixin on TBARS and antioxidant enzymes activities in kidney

Parameters	Control	AAP	GGJ+AAP	Nb+AAP	CCJ	Nb
			Kidney			
TBARS nmol/ mg protein	0.49 ± 0.02	0.57 ± 0.03[a]	0.53 ± 0.04	0.54 ± 0.04	0.49 ± 0.02[b]	0.50 ± 0.03[b]
Total SOD U/ mg protein	29.39 ± 1.18	20.64 ± 2.19[a]	21.18 ± 1.83[a]	31.57 ± 3.25[b]	27.92 ± 0.89[b]	35.35± 1.28[ab]
MnSOD U/ mg protein	10.16 ± 0.34	12.14 ± 1.68[a]	9.29 ± 0.58[b]	11.14 ± 1.63	11.01 ± 0.20	13.51 ± 0.37[a]
CuZnSOD U/ mg protein	19.22 ± 1.14	8.51 ± 0.63[a]	11.89 ± 1.48[ab]	20.43 ± 3.27[b]	16.91 ± 0.94[b]	21.84 ± 1.14[b]
Total GPx U/ mg protein	10.58 ± 0.31	10.49 ± 0.39	4.08 ± 0.31[ab]	5.33 ± 0.23[ab]	10.32 ± 0.49	7.80 ± 0.45[ab]
GPx-se-dependente U/ mg protein	8.02 ± 0.40	5.27 ± 0.28[a]	2.30 ± 0.32[ab]	0.89 ± 0.06[ab]	4.80 ± 0.31[a]	5.94 ± 0.35[ab]
Catalase pmol/ mg protein	0.86 ± 0.07	0.81 ± 0.05	1.64 ± 0.04[ab]	0.75 ± 0.03[a]	0.24 ± 0.03[ab]	0.21 ± 0.03[ab]

Results are mean ± S.D., n=5. AAP- acetaminophen; CCJ- Concord grape juice; Nb- Norbixina.
[a] Significantly different from Control, p≤ 0.05. [b] Significantly different from AAP, p≤ 0.05.

3.2 Antioxidant enzymes activities

AAP group showed distinct responses in liver and kidney antioxidant enzymes activities, except for the catalase activities, which were not affected by the AAP treatment in both liver and kidney (Table-1-2). The liver from AAP group exhibited a significant increase ($p \leq 0.05$) in the Total SOD and CuZnSOD activities when compared to the Control group. However, in kidney, both Total SOD and CuZnSOd activities were decreased significantly ($p \leq 0.05$). After AAP treatments liver did not occur the reduction of GPx activity, contrarily the Total GPx activity was significantly increased ($p \leq 0.05$) in 30% when compared to Control group. In kidney Total GPx activity was not altered, but GPx Se-dependent was significantly decreased ($p \leq 0.05$) when compared to the Control group.

Total SOD and CuZnSOD activities in the CGJ+AAP group were not changed in the liver and kidney when compared to AAP group, but in kidney the MnSOD activity was decreased significantly ($p \leq 0.05$). Liver and kidney showed decrease significantly ($p \leq 0.05$) in Total GPx and GPxSe–dependent activities when compared to AAP group. The catalase activity showed an increase of 200% in liver and 100% in kidney when compared to AAP group ($p \leq 0.05$) (Tables 1-2).

CuZnSOD activity did not change in the liver Nb+AAP, however MnSOD increased in comparison to the Control and the AAP groups ($p \leq 0.05$) (Table-1). In the kidney Nb+AAP showed an increase in total SOD and CuznSOD when compared to AAP group ($p \leq 0.05$). The total GPx and GPx Se-dependent activities, in both liver and kidney, decreased significantly ($p \leq 0.05$) in comparison to AAP group. Liver Nb+AAP catalase activity were increased in 54% when compared to AAP group, meanwhile the kidney did not demonstrated increases in catalase activity in comparison to AAP group.

CGJ tested alone (Table-1) did not demonstrate alterations in Total GPx in the liver, meanwhile Total SOD, CuZnSOD and MnSOD were increased significantly ($p \leq 0.05$) when compared to the Control group ($p \leq 0.05$). In kidney, Total GPx, Total SOD, CuZnSOD and MnSOD did not changed when compared to the Control group ($p \leq 0.05$) (table-2). GPx Se-dependent was reduced significantly in liver while in the kidney it was

increased significantly ($p \leq 0.05$) in comparison to the Control group. The catalase activities were reduced in both liver and kidney.

Nb tested alone (Tables-1-2) demonstrated similar responses in liver and kidney, increased significantly ($p \leq 0.05$) the Total SOD and MnSOD activities, and decreased Total GPx, Gpx-Se-dependent and catalase, in both organs.

3.3 Histologycal analysis

The Figure 1 shows Hematoxilin-eosin stained micrographs from Ligh microscopy (LM). The figure 2 shows micrographs from Transmission Electronic Microscopy.

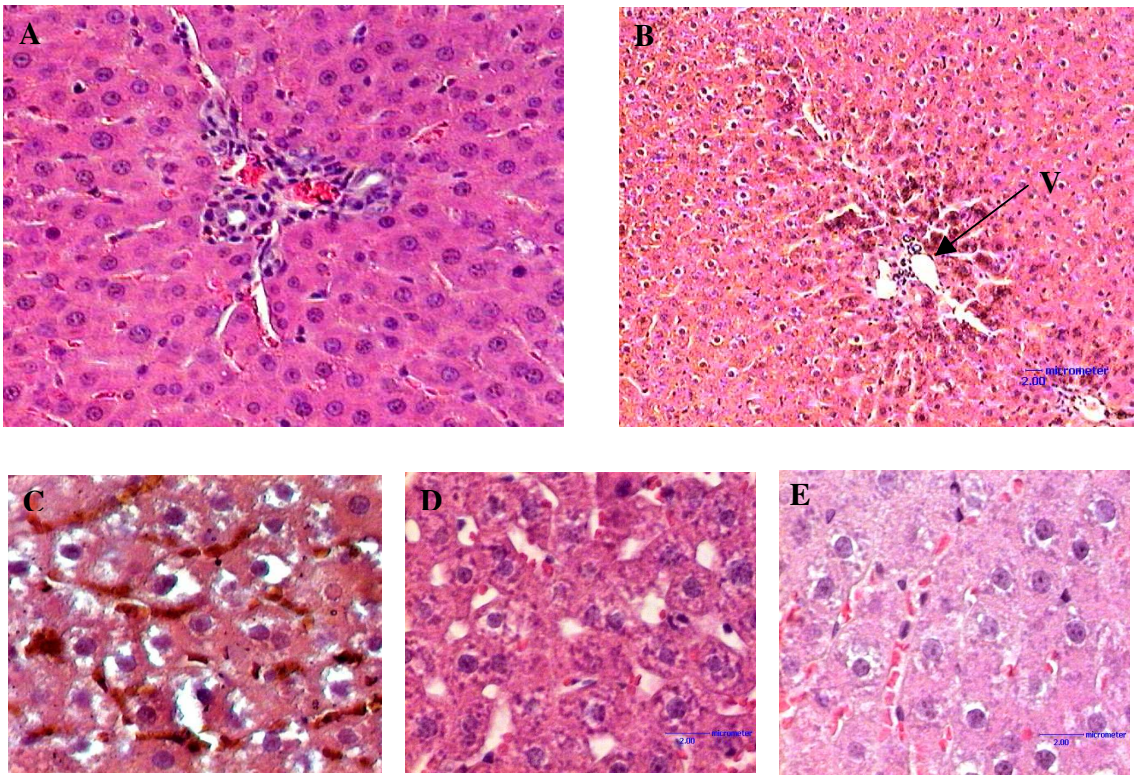

Figure 1. A- Control liver, characterized by the homogeneous cytoplasm and normal chromatin disposition inside the nuclei; B – Liver cells altered after *in vivo* AAP administration, are characterized by the all over picnotic nuclei. The darker deposits in cytoplasm, are mostly present in the cells around the venules (V); these dark pigments demonstrates organelles disruption; C - These altered liver cells after in vivo AAP administration under DAB histologycal reactions indicates the high disruption of peroxiomes bodies; D - AAP+CCJ – The previous daily ingestion of CGJ following the AAP administration revels a rather functional liver cells, despite the cytosolic organelles aggregation; E - AAP+Nb, the hepatic tissue it is preserved nevertheless the vacuole around nuclear structure.

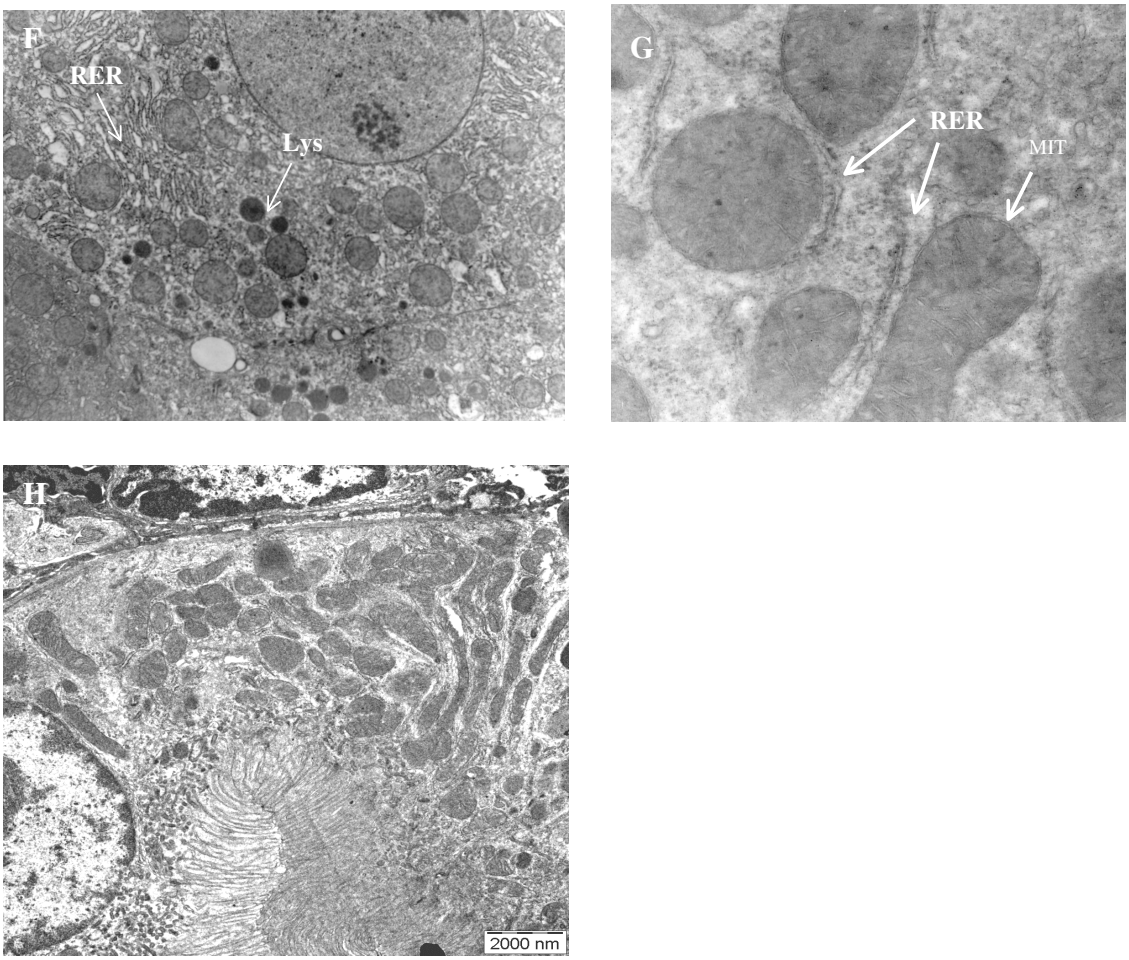

Figure 2. F - Nb+AAP as mentioned above (E), this treatment preserved the liver tissues nevertheless the RER engulfment; G- CGJ alone did not affect any cellular structures. These slim RER it is on physiological state as well as the mitochondria; H- Nb alone was inocuous to liver, and in the kidney did not affect the glomeruli and proximal tubule as it is shown here. However distal tubule cells initiated lysosome disruption, what indicates that Nb has a specific and punctual disturbance under the employed dose.

4. Discussion

4.1 Lipid peroxidation

Consistent to the oxidative stress theory of AAP toxicity (GIBSON, 1996), in our study the TBARS levels were increased in AAP-treated rats. The previous administration of CGJ or Nb followed by acetaminophen, reduced the TBARS levels in liver and in kidney occurred a lower reduction. CCJ contain many polyphenolic compounds and their hydroxyl groups could donate hydrogen atoms at initial stage of lipoperoxidation, therefore inactivate hidroperoxides formed from poly unsatured fatty acids (PUFAs), and thus broking chain propagation; besides this, the phenolic compounds can induce the antioxidant enzymes activity (AHN et al, 2002; JODYNIS- LIEBERT et al, 2005). It has been previously demonstrated that bixin function as a quencher of singlet oxygen and scavenger superoxide anion and this fact could to contributed to the TBARS levels reduction of these animals. (SANTOS et al., 2002; DE-OLIVERA, 2003). The lower TBARS levels reduction in the kidney can due to the fact that this compounds act primarily in liver and consequentially lower concentrations of the antioxidants would arrive to the kidney and other organs. Furthermore biotransformations can occurs during the passage through the liver and could produce less actives metabolites than initial compounds.

4.2 Antioxidants enzymes activities

Acetaminophen is able to increase the oxidative stress in the liver therefore it is a critical factor of AAP-induced injury. Previous studies appoint the increased in superoxide radical as the mechanism of hepatic AAP-toxicity (MIROCHNITCHENKO, 1999). For example, LOREZ ARNAIZ et al., 1995 found an increased in superoxide radical more than 2-fold in mouse liver following a large dose of AAP. The increased in the liver total SOD and CuZnSOD due to AAP administration could be due to its induction as protective responses to superoxide radical production increased during AAP hepatic metabolism. The increase in SOD activity generates the higher production of H_2O_2, which will be detoxified by the catalase or GPx enzymes. Without pertinent reduction of H_2O_2, by these enzymes, it might react with metallic ions, such as the cellular iron, generating highly reactive hydroxyl radicals. The increased in the liver GPx activity suggests an induction by both higher

organic and inorganic peroxides. SHULL et al., 1991 reported an increase in GPx RNAm at higher concentrations of H_2O_2 in epithelial cells.

Toxics metabolites from acetaminophen and/or peroxides may be released from the liver to the blood and then affect other organs. It is also known that antioxidants enzymes can be inactivated by excess of lipid hidroperoxides and ROS. SOD can be inhibited by hydrogen peroxide, GPx and CAT by an excess of superoxide radical. (MATÉS & SÁNCHEZ-JIMÉNEZ, et al., 1999; JODYNIS- LIEBERT et al, 2005). The excess of metabolites such hidroperoxides can explain the observed decrease of SOD and GPxSe-dependent activities in the kidney.

Previous treatment with grape juice followed to AAP, stimulates the catalase activity in both liver and kidney; therefore could increase the H_2O_2 neutralization avoiding OH^- production and as consequence there is a reduction in TBARS. FKI et al, 2005 demonstrated similarly increase in liver CAT activity in animal treated with green and black olives phenolic extracts.

Norbixin increased the SOD activity in AAP- treated rats, attenuating the AAP effects. However, the GPx enzymes did not followed the SOD increase and in kidney catalase activity also not changed. It is well known that the ROS scavenging SOD activity is effective only if is followed by the CAT and GPx, activities; the dismutase SOD activity generates H_2O_2, which needs to be further scavenged by CAT and GPx. This explains the present results where the TBARS levels reduction were greater in the livers than in the kidneys (LEE et al, 2003).

CCJ or Nb tested alone did not changed the TBARS levels, but induced modifications in antioxidants enzymes activities. These modifications, in absence of oxidative stress, reinforces the viewpoint that the polyphenols from CGJ and Nb serves as quencher, but also can act as signaling molecules, therefore modulating the expression of this antioxidant enzymes.

4.3 Histological analisys

Liver and kidney histology by TEM and LM analysis, demonstrated as other studies, that AAP chanded the structure of these organs (MIROCHNITCHENKO, 1999,

WATERS, 2001). These results are in agreement with the enzymatic analyses. AAP liver showed oxidant disruption along capillary vessels and under DAB test (figure 1-C) reveals peroxisomes lesions and citoplasmatic disruption. The kidney under AAP treatment demonstrated lesions mostly over distal tubules.

Liver and kidney of CCJ+AAP depicted apparent normality. CCJ alone do not altered the tissue structure (Figure 2-G). These results follow the same enzymatic analysis responses.

A Norbixin dose administered was somewhat deleterious to the renal lysosome distal tubules. Therefore it was not surprising that Nb + AAP (figure 2-H) still demonstrated alterations especially to the RER and lysosomes structures.

5. Conclusions

Our results demonstrated that Concord grape juice and Norbixin attenuated the effects of AAP induced liver and kidney injury. Both antioxidants presented distinct responses in liver and kidney

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CAPÍTULO 5: CONCLUSÃO GERAL

Conclusão Geral

Os resultados obtidos através das análises *in vitro*, confirmaram que os sucos de uva são expressivas fontes de fenólicos totais, sendo que o suco concentrado Concord apresenta teores comparáveis ao vinho tinto. Este suco Concord também apresentou o composto piacetanol-O-glicosídeo, uma fitoalexina da mesma família do resveratrol, ao qual é atribuído grande potencial antioxidante. Os sucos analisados apresentam alta capacidade antioxidante a qual se mantém durante as etapas de produção e estocagem, especialmente sob refrigeração.

As análises *in vivo* mostram que o suco de uva Concord e a Norbixina diminuem os danos oxidativos, neste estudo, provocados pelo acetaminofeno. Os resultados obtidos demonstram que em situações onde ocorra a presença de estresse oxidativo, estas substâncias antioxidantes (CGJ e Nb) estimulam o sistema enzimático de defesa antioxidante. Neste estudo o tratamento prévio com CGJ e Nb seguido pela administração de acetaminofeno aumenta expressivamente a atividade da enzima catalase, contribuindo assim para a redução de danos oxidativos, especialmente a peroxidação lipídica. Outro achado importante do estudo foi à confirmação de que tais substâncias antioxidantes atuam de maneira diferenciada nos tecidos corporais, visto as diferentes respostas enzimáticas apresentadas por fígado e rins.

ANEXOS

ANEXO 1 – Certificado de aprovação pelo comitê de ética em experimentação animal.

UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1129-1**, sobre **"Resposta hepática e renal à administração de antioxidantes naturais em animais submetidos à oxidação por paracetamol (Acetaminophen)"**, sob a responsabilidade de **Profa. Dra. Débora de Queiroz Tavares / Jane Cristina de Souza**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 29 de novembro de 2006.

CERTIFICATE

We certify that the protocol nº **1129-1**, entitled **"Natural antioxidants in preventing acetaminophen-induced hepatic and kidney injury in the rat"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on November 29, 2006.

Campinas, 29 de novembro de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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ANEXO 2

Trabalho desenvolvido durante o curso de mestrado como colaboradora.

Gollücke, A. P. B.; Souza, J. C.; Tavares, D. Q. Sensory stability of Concord and Isabel concentrated grape juices during storage. Artigo aceito para publicação no **Journal of Sensory studies.**