Cyclic voltammetric analysis of antioxidant activity in citrus fruits from Southeast Asia

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Abstract: The aims of this work were principally two-fold: first, to show that cyclic voltammetry is a simple, cheap and practical method for determining antioxidants in citrus fruit juices; second, to evaluate vitamin C in four species of citrus fruits. Two are known world wide, the lemon, *Citrus limon* (L.) Burm.f., and the lime, *Citrus aurantiifolia* (Christm.) Swingle, and two are principally but not exclusively found in Southeast Asia where they are of economic importance, the sweet lime, known in Malay as *limau manis* and the musk lime, *limau kasturi*. Juices from all the citrus fruits contained antioxidants with the principal one being equivalent to vitamin C at concentrations of 1.3 mM in *limau manis*, 1.4 mM in *limau kasturi*, 1.5 mM in *limau kapas* and 1.8 mM in lemon. It was also evident that the citrus fruit juices contained one or two additional antioxidants, although their presence was variable. The use of differentiated recording gave greater resolution of the additional antioxidants.

Keywords: cyclic voltammetry, antioxidant, vitamin C, citrus fruit, lemon juice, lime juice, *limau kasturi*, *limau manis*

Introduction

Increasing awareness of the role of the importance of antioxidants in the diet has led to greater importance placed on the consumption of foods containing antioxidants (Vita, 2005; Valdés, 2006; Li and Schellhorn, 2007). Not least within the food industry ascorbic acid, vitamin C, is important as a dietary component and as a food preservative. The dietary value of oranges and lemons or their juices is long established, having been determined by evidence-based medicine in the eighteenth century to be antiscorbutic (Krehl, 1953). The isolation of vitamin C from lemon juice led to its original identification (King and Waugh, 1932; Jukes, 1988). The beneficial effects of vitamin C are not restricted to its antiscorbutic activity as there is now evidence to show that it is protective against neurodegeneration and may ameliorate some cardiovascular diseases and have anti-cancer properties (Li and Schellhorn, 2007).

Determination of vitamin C as an analyte can be problematic because of its oxidation to dehydroascorbic acid. In a clinical setting, because dehydroascorbic acid can be oxidised back to vitamin C, it too is of value and, for example, total plasma ascorbate concentrations may be more important than those of vitamin C alone (Margolis and Duewer, 1996). Determination of vitamin C is most accurately performed by chromatographic separation and determination by electrochemical or spectroscopic detection (Margolis and Duewer, 1996). Colourimetric assays, using iodine-starch or 2,6-dichlorophenolindophenol (DCPIP), although they have been useful in the past and are still used (Guerrant et al., 1935; Suntornsuk et al., 2002; Abbasi and Niakousari, 2008; Zvaigzne et al., 2009) may not be the most reliable (Margolis and Duewer, 1996).

Differential pulse voltammetry for the determination of vitamin C has been described as simple and rapid (Ballantine and Woolfson, 1980; Barthus et al., 2005); square-wave voltammetry coupled to an electrochemiluminescence technique has been described for quantitation of vitamin C and dehydroascorbic acid (Takahashi and Jin, 2009). Here we describe the use of cyclic voltammetry for analysing vitamin C.

The aims of this work were principally two-fold: first, to show that cyclic voltammetry is a simple, cheap and practical method for determining antioxidants in citrus fruit juices; second, to evaluate vitamin C in four species of citrus fruits. Two are

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known world wide, the lemon, *Citrus limon* (L.) Burm.f., and the lime, *Citrus aurantiifolia* (Christm.) Swingle, and two are found in Southeast Asia, the sweet lime, known in Malay as *limau manis* and the musk lime, *limau kasturi*.

Citrus fruit taxonomy is complicated because their plants readily reproduce asexually with accompanying somatic diversity, and they also readily hybridise (De Araujo et al., 2003). Possibly there are only three true species of *Citrus*, the pomelo, *C. grandis*, the mandarin, *C. reticulata* and the citron, *C. medica* (Moore, 2001). Several authorities describe the *limau manis* (sweet lime, sweet orange) as *Citrus sinensis* (L.) Osbeck (for example (ITFN, 2009)), which is the also the specific name for the common orange. It is possible that *limau manis* is a variety or cultivar of *C. sinensis*, but its general appearance does not fit with the usual descriptions of this species. The skin is thin, about 2 mm and its colour is green, sometimes blotched with orange. The flesh is orange and can taste sweet, but its flavour tends to be orange-like and insipid. It is eaten fresh and is not particularly used for culinary purposes, nor is it juiced for beverages. *Limau kasturi* is known variously as *Citrus mitis* Blanco, *Citrus microcarpa* Bunge, *Citrus madurensis* Lour, and as a hybrid *Citrofortunella* x *mitis* (Blanco) Ingram and Moore (IPNI, 2009; ITFN, 2009; ZipcodeZoo, 2009) or as a hybrid of *C. reticulata* (Blanco) x *Fortunella* spp. (Nisperos-Carriedo et al., 1992). It has a sharpness that is perhaps not quite a strong as lemon or lime and is not generally palatable enough to be eaten fresh. However it has a good flavour, somewhat orange-like, somewhat lime-like, and in Southeast Asia it is used in the preparation of beverages (blended with ice or warm water, with added sugar) and is widely used in food preparation either as a condiment or as an ingredient. It is also used commercially to make soft drinks under its common names of kalamansis, calamansis or calamondin. Both *limau kasturi* and *limau manis* are widely available in local markets in Brunei and its neighbouring Malaysian states of Sabah and Sarawak. The kasturi lime is also widespread in the Philippines and Indonesia. In these Southeast Asian countries these citrus fruits are economically important.

The lime used in this study is also common in Southeast Asia, and is known locally in Brunei as *limau kapas*. Lemons are not grown commercially in Brunei and the ones used in this study were imported from China. The *limau kapas* and lemon were used for comparison with the two native *limau* because there is comparative information in the literature about their antioxidant and vitamin C content. To our knowledge there is no available information on the antioxidant or vitamin C content of *limau manis* or *limau kasturi* grown in Brunei Darussalam.

Materials and Methods

Vitamin C standards

Vitamin C (ascorbic acid) was used as a standard antioxidant for comparison of the fruit juices. Cyclic voltammograms were constructed for vitamin C at concentrations from 5.1 µM to 0.51 mM. Vitamin C (Krüger GmbH and Co., Germany) dilutions were prepared at the start of the experiments either from fresh or from frozen (-20°C) aliquots of vitamin C (51.1 mM). The vitamin C dilutions prepared from a frozen stock yielded responses that were not distinguishable from those from a freshly prepared stock.

Citrus fruits

Limau kapas (*Citrus aurantiifolia* (Christm.) Swingle), *limau kasturi* (*Citrofortunella* x *mitis* (Blanco) Ingram and Moore), and *limau manis* (*Citrus sinensis* var) were bought from local markets, and lemons (*Citrus limon* (L.) Burm.f.) were bought from a supermarket. Fruits were cut in half, squeezed by hand and strained to remove pips, pulp and sacs. The juice was diluted in phosphate buffer, (50 mM, pH 6.2, room temperature). Each fruit juice was tested over a concentration range from 0.1% to 10% (v/v juice/buffer). Each individual sample concentration was tested twice, and seven of each type of fruit was tested. *Limau manis*, *limau kapas* and *limau kasturi* are illustrated in Figure 1.

Cyclic voltammetry

Cyclic voltammetry was performed using an eDAQ system (www.eDAQ.com), consisting of an EA161 potentiostat connected to an e-corder which inputted to eChem software (running on a PC using Microsoft Windows Vista platform). The working electrode was 3-mm diameter glassy carbon; the reference electrode was Ag/AgCl in 3 M NaCl (+207 mV vs. standard hydrogen electrode); the auxiliary electrode was 0.25-mm diameter Pt wire. The volume of the voltammetric cell was approximately 15 mL. The working electrode was polished for at least 4 min before individual cycles of voltammetry. In pilot experiments the diapason of the potential was -1 to +1.5 V, but routinely applied potentials ranged between -200 mV to +1000 mV because there did not appear to be any information outside this range. The scan rate was 200 mV.s^{-1}.

The relationship between peak anodic current (Ipa) and concentration of vitamin C or fruit juice
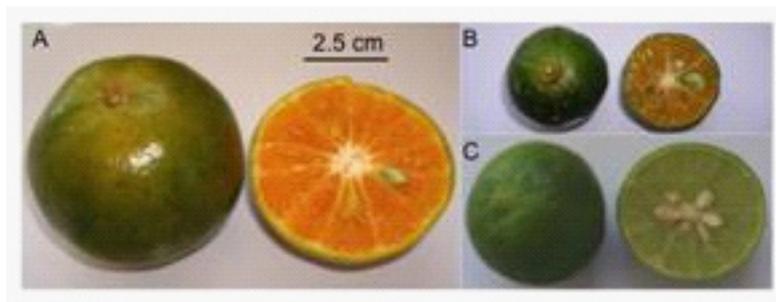
Figure 1. Southeast Asian citrus fruits. A. *Limau manis* (sweet lime or sweet orange, possibly a variety of *Citrus sinensis*), B. *Limau kasturi* (musk lime, calamansis, *Citrofortunella* x *mitis* (Blanco) J. Ingram & H.E. Moore), C. *Limau kapas* (*Citrus aurantiifolia* (Christm.) Swingle).

was determined. The voltammograms were also subjected to first order differentiation with respect to time (using eChem software). The resulting transformed voltammograms give $(d/dt)I_{pa}$ with a peak corresponding to the fastest rate of rise of the I_{pa} or the rate of oxidation of the antioxidant (Sia et al., 2010). Double reciprocal plots of I_{pa} and concentration were also used if necessary; they provide a linear transformation of non-linear concentration-response relationships (Sia et al., 2010).

For vitamin C, the limits of detection and quantification were estimated from the lowest concentration assayed (5.1 µM) using 3σ and 10σ, respectively (Armbruster et al., 1994; Armbruster and Pry, 2008).

Results and Discussions

Vitamin C standards

Over a range of 5.1 µM to 0.51 mM, vitamin C produced a single concentration-dependent peak anodic current (I_{pa}). The mean potential (E_{pa}) at which I_{pa} occurred was between 156 ± 15 mV at 5.1 µM and 187 ± 19 mV at 1.02 mM (n = 7 independent samples measured in duplicate at each concentration). There were no observable peak cathodic currents (I_{pc}).

The relationship between I_{pa} and concentration of vitamin C was linear (Figure 2A), with an equation of $I_{pa} = 37.7$[vitamin C] $+ 2.19$ with a coefficient of determination (r^2) of 0.999. The intercept was not significantly different from zero. The differentiated voltammograms also yielded a linear relationship between the rate of oxidation and concentration of vitamin C (Figure 1B). The regression was $(d/dt)I_{pa} = 81.6$[vitamin C] $- 0.21$ with a coefficient of determination (r^2) of 0.998. The intercept was not significantly different from zero. The relationship between Ipa and $(d/dt)I_{pa}$ was linear (Figure 2C) with a coefficient of determination (r^2) of 0.998. The

intercept was not significantly different from zero.

At a concentration of 5.1 µM, vitamin C gave a signal of 0.22 ± 0.083 µA (mean $\pm \sigma$, n = 7), leading to an estimated limit of detection of 6.6 µM and a limit of quantitation of 22.0 µM. From the differentiated recordings the signal was 0.36 ± 0.076, leading to a limit of detection of 2.8 µM and limit of quantitation of 9.3 µM.

Fruit juices

For all the fruit juices there was always a major peak that arose between 254 and 574 mV. The amplitude of the peak anodic current was concentration-dependent (Figures 3 and 4). This peak was observable reliably at concentrations of 0.3 % and above for lemon juice, *limau kapas* and *limau kasturi*, but for *limau manis* it was only reliably present at 1% or above. In addition there were low-amplitude peaks that occurred at higher potentials, but these were not always present. They were seen best at intermediate concentrations because they were not large enough to be seen at lower concentration, and at higher concentrations they were often obscured by the main peak. These lesser peaks were not analysed in any detail. However, in most samples the presence of additional compounds was more evident in the first order derivative traces (Figure 4 insets).

For all the citrus fruit juices the relationship between I_{pa} and concentration was linear, with highly statistically significant regressions (Figure 3). For limau manis, at 10% the I_{pa} was not as great as might be expected, and the concentration-response relationship appeared to plateau. When the double reciprocal plot was constructed the relationship became linear with a regression coefficient (r^2) of 0.999 from three degrees of freedom, and an intercept not significantly different from zero.

The slope of the regression of I_{pa} on concentration for all the citrus fruit juices was interpolated to the
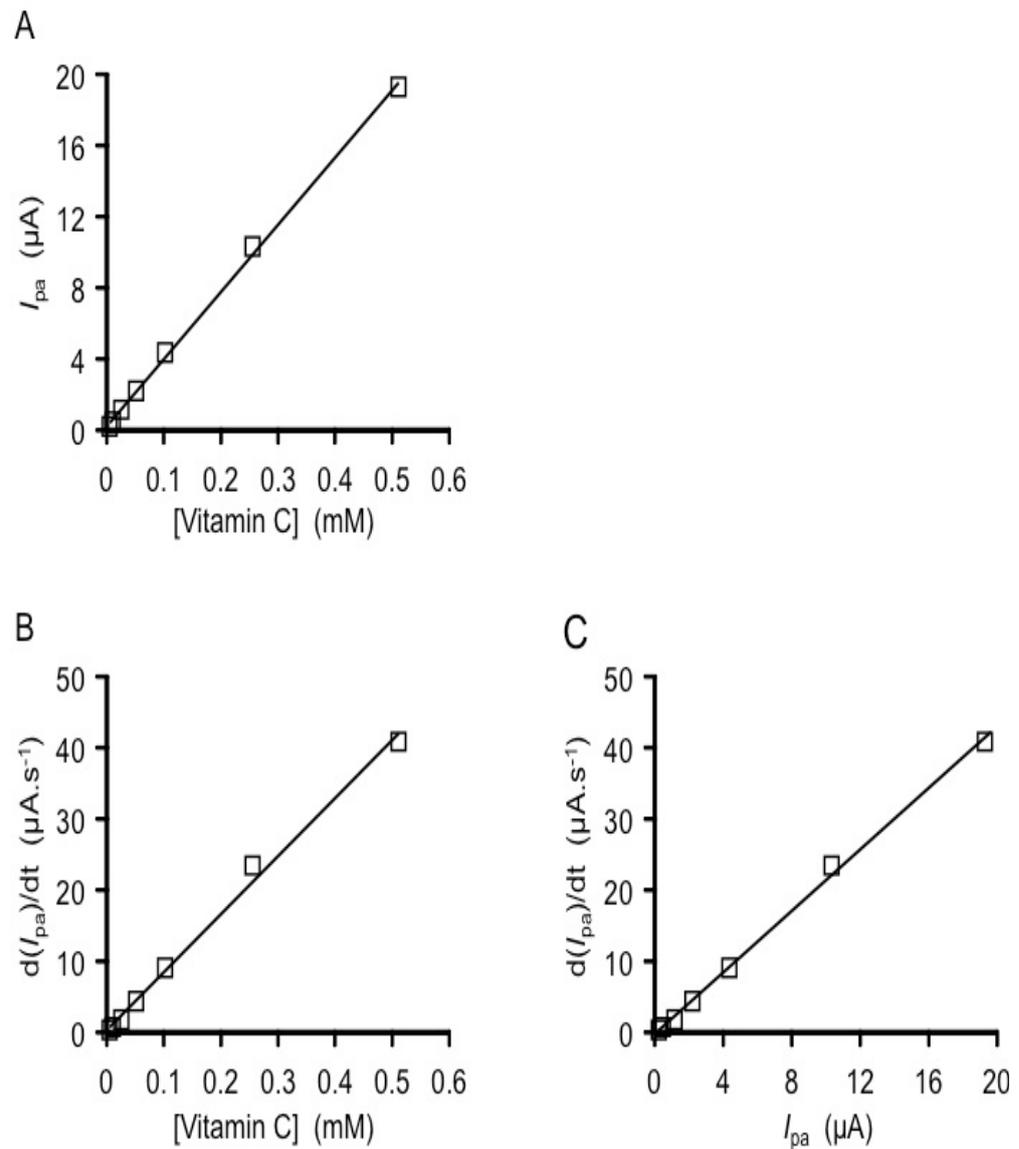
Figure 2. Characteristics of vitamin C by voltammetry. A) Vitamin C (5.1 μM to 0.51 mM) produced a concentration-dependent linear increase in peak anodic current (I_{pa}). B) The first order derivative of the I_{pa} also has a linear dependency on concentration. C) The first order derivative has a linear relation with the I_{pa}. All point show the mean, bars show standard error of the mean, unless occluded by the symbol. All points, n = 7 independent samples, each concentration of each sample was measured in duplicate.
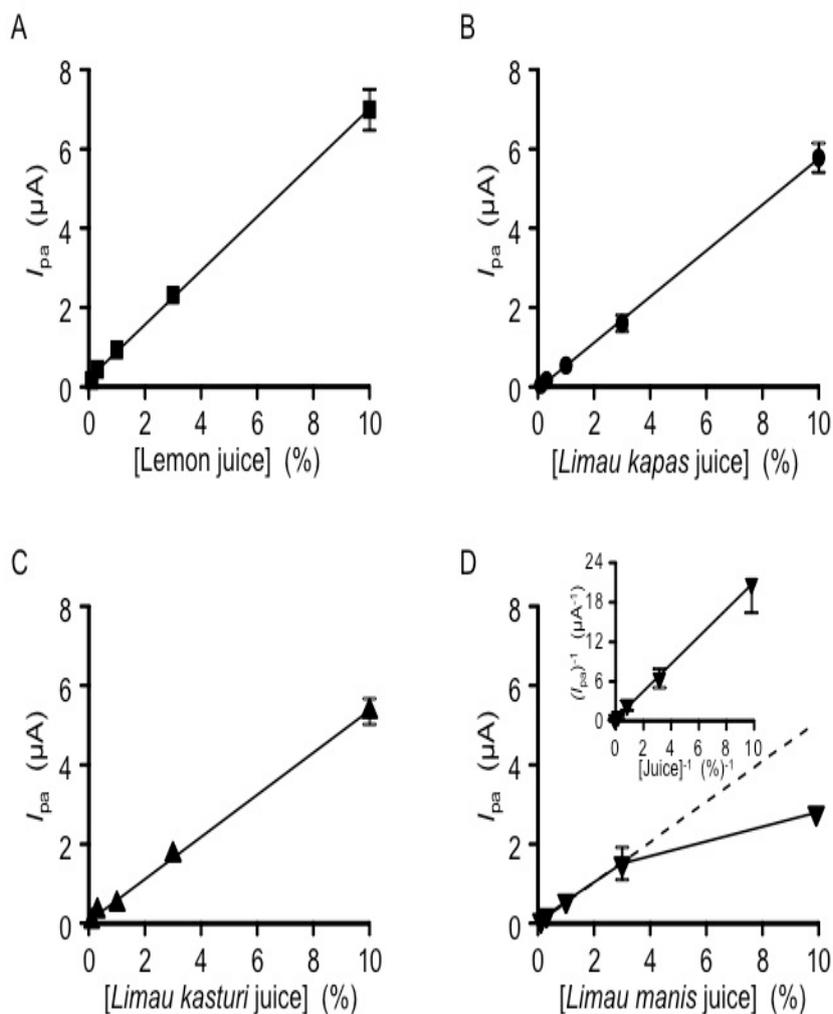
Figure 3. Peak anodic currents in relation to concentration in citrus fruit juices. A) Lemon juice, B) *Limau kapas*. C) Limau kasturi. D) Limau manis. In A, B and C the relationship is highly statistically significantly linear, all with a coefficient of determination (r^2) of 0.998 or 0.999, the equations being 0.68x + 012, o.58x + 0.08 and 0.53x + 0.01 for lemon, limau kapas and limau kasturi, respectively. In D the relation is more significantly linear for concentrations from 0.1 to 3%; the dashed line is the regression of these points; inset is the double reciprocal plot of all points, which has a statistically significant linear regression of $1/I_{pa}$ = 2.1[limau manis juice] + 0.17, r^2 = 0.999, 3 degrees of freedom; the intercept does not differ significantly from zero. In all panels the points show mean and bars show standard error unless occluded by the symbol from seven independent samples, each concentration of each sample was measured in duplicate.
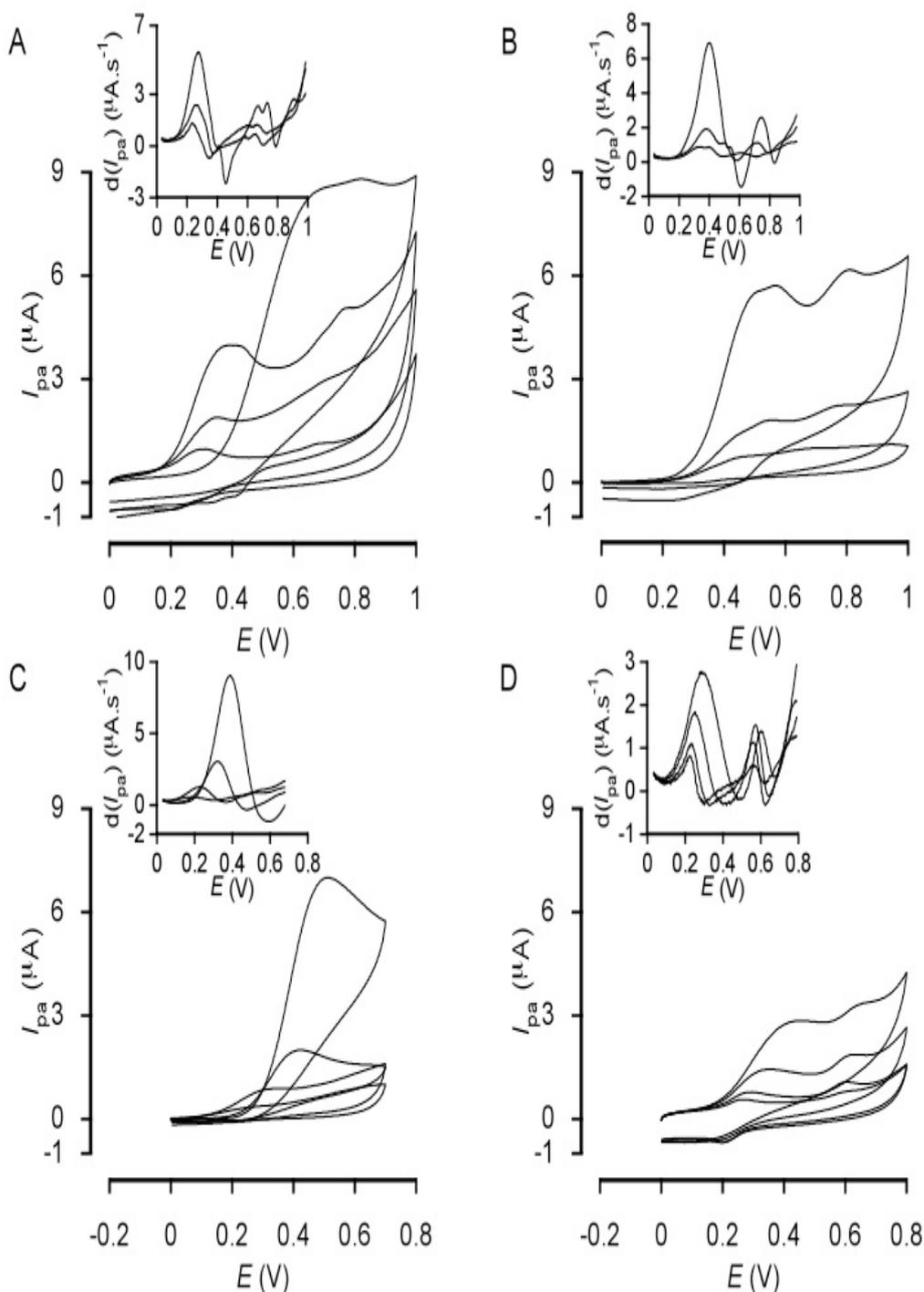
Figure 4. Cyclic voltammograms of citrus fruit juices. Cyclic voltammograms for fruit juices diluted (0.1 to 10 % v/v) in 0.1 M phosphate buffer, pH 6.2. The background (phosphate buffer alone) has been subtracted.
A) Lemon juice, the superimposed voltammograms are at 0.3, 1, 3 and 10%. Inset are the differentiated voltammograms for 0.3, 1 and 3 %. Note in the differentiated traces the appearance of two small peaks between 0.6 and 0.8 V which are less evident in the anodic current recordings. B) *Limau kapas*, 1, 3 and 10 %. Note that the peak between 0.4 and 0.6 V is broad and irregular. In the differentiated recordings (inset) this peak can be resolved into two separate peaks at the two lowest concentrations. Note also the presence of a third peak around 0.8 V which is more evident in the differentiated recordings than in the anodic currents recordings. C) *Limau kasturi*, in this example there was only one observable peak at concentrations of 0.3, 1, 3 and 10%. Note the rightward shift in potential with increasing concentration of juice. D) *Limau manis*, 0.3, 1, 3 and 10%. The peak occurring between 0.2 and 0.4 V appears to be clean and there is also a peak around 0.6 V which is clearer in the differentiated recordings than in the anodic current recordings.

same regression for vitamin C standards, to give the vitamin C equivalent for the content of the fruit juice represented by the major peak. For *limau manis* this regression was restricted to concentrations from 0.1 to 3% (Figure 3), but the same value of vitamin C equivalent was obtained when the slope of the double reciprocal transformation (Figure 3D inset) was used to derive the vitamin C equivalent. The rank order of vitamin C equivalents in the citrus fruit juices was: lemon, 1.8 ± 0.10 mM $>$ *limau kapas*, 1.5 ± 0.09 mM $\geq$ *limau kasturi*, 1.4 ± 0.08 mM $\geq$ *limau manis*, 1.3 ± 0.08 mM.

The results show that all the fruit juices analysed contain antioxidants, with the principal antioxidant most likely being vitamin C. The concentration of the presumed vitamin C was greatest in lemon juice and least (approximately 28% lower) in *limau manis*. All the fruit juices contained additional substances that could be electrochemically reduced at positive potentials below 1 V. These components appeared to be minor compared to vitamin C; their appearance was variable amongst samples of fruits. *Limau kasturi* juice contained fewest or least significant additional compounds while *limau kapas* in some samples contained up to three additional compounds. Usually it was possible to identify, if not quantify, one or two additional compounds in juice from lemon, *limau kapas* and *limau manis*.

The vitamin C standards gave a range of anodic current signals that encompassed the range of signals from the fruit juices. The linear relation between the differentiated signal and the concentration of vitamin C, and also between the differentiated signal and the I_{pa} itself, show that this parameter, $d(I_{pa})/dt$, can equally be used in analysis of voltammograms, as we have previously shown (Sia et al., 2010). The limits of quantitation and detection were approximately three orders of magnitude below the concentrations determined in any of the fruit juices.

It is well known that citrus fruit juices contains vitamin C. An early evaluation of the vitamin C content of fresh lemon juice was 0.5 mM (Smith and King, 1931) and using iodate or DCPIP colourimetric assays, the concentrations determined were around 3.2 and 3.7 mM for fresh lemon and orange juice (Bessey and King, 1933), 2.6, 2.6 and 2.8 mM for fresh lemon, lime and orange juice (Guerrant et al., 1935) and 3.9, 2.6 and 3.7 mM in fresh lemon, lime and orange juice (Burton, 1937), all values respectively. The colourimetric assays overestimate the concentration of vitamin C because other antioxidants can contribute to the colour-change reaction (Bennett and Tarbert, 1933; Bessey and King, 1933; Guerrant et al., 1935; Burton,

1937). In a comparative study of methodologies, the DCPIP assay has been shown to overestimate the concentration of vitamin C (Margolis and Duewer, 1996). More recent determinations for lemon (*C. limon*) juice are: 1.3 mM and 2.2 mM (by HPLC, (De Carvalho et al., 2006; Artés-Hernández et al., 2007)), and 2.2 mM and 2.5 mM (by DCPIP, (Abbasi and Niakousari, 2008; Zvaigzne et al., 2009)); for lime (*C. aurantiifolia*) juice, using HPLC, 1.5 mM (Theansuwan et al., 2008), and using DCPIP 0.6 mM (Suntornsuk et al., 2002); for orange juice 3.2 mM (Zvaigzne et al., 2009) and 2.8 - 3.1 mM (Noroozifar and Khorasani-Motlagh, 2003) using HPLC and DCPIP or a novel colorimetric assay, respectively. The values for lemon, lime and orange juice in the USDA National Nutrient Database for Standard Reference are equivalent to 2.6, 1.7 and 2.8 mM, respectively (UNND, 2009). In the Malaysian Foods Composition Database, values equivalent to 1.75 to 2.3 mM are given for *limau kapas*, and 1.3 to 2.4 mM for *limau kasturi* (MFCD, 2009). In one study, using HPLC the vitamin C concentration in *limau kasturi* juice was 2.6 mM (Nisperos-Carriedo et al., 1992).

The values obtained in the current study for lemon juice seem to be much lower than most literature values, although they are higher than the values found in lemons from Brazil (De Carvalho et al., 2006). The lemons used in this study were imported from China and no information was available as to how much time had elapsed since harvest, however, Bennett and Tarbert (1933) noted "Even more striking variations were found between individual lemons. Twenty lemons gathered from the same tree on March 2nd and squeezed separately gave results varying from 4.9 to 10.0." The vitamin C equivalent determined in the present study, of 1.8 mM is on the low side, but may not be abnormal. The value of 1.5 mM obtained for *limau kapas* is consistent with literature values for limes, *C. aurantiifolia*. The vitamin C equivalent of Limau kasturi is close to that of limau kapas and is within the range given in the Malaysian Foods Composition database. Compared to orange (*C. sinensis*) juice, the concentration of vitamin C in *limau manis* juice is very low. If this *limau manis* is indeed a variety or cultivar of *C. sinensis* it is unusual for this species in having such a low vitamin C content, only one half to one third of that found in other varieties.

Although we have described the predominant antioxidant in the citrus fruit juices in terms of vitamin C equivalents, it is more than likely that the peak anodic currents measured do represent vitamin C even though the potential at which they occur is higher than that for authentic vitamin C. It is well

established that citrus fruits contain vitamin C, and the vitamin C has to be represented by these peak anodic currents under these recording conditions, because there is no other peak that could represent it. The material analysed was complete fruit juice and it is assumed that other components cause the currents for the impure vitamin C to occur at the slightly higher potentials than authentic vitamin C in isolation (Takahashi and Jin, 2009). Indeed, for *limau manis* at the highest concentration tested there was evidence for some adsorption onto the electrode surface resulting in a concentration-response relationship that deviated from a straight line towards a form that appeared to plateau.

In conclusion, cyclic voltammetry can be used to analyse citrus fruit juices for antioxidant activity and vitamin C. The method is clean and simple to set up and use and has some advantages over HPLC or colourimetric assays in that it does not require any reagents apart from a simple buffer solution, and is cheaper and more time-efficient. Furthermore, at the same time other antioxidants can be seen in the fruit juices, which although they were not examined in any detail in this study could be interesting compounds to investigate.

Acknowledgements

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