

Electrochemical bromination of organosulfur containing species for the determination of the strength of garlic (*A. Sativum*)

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ABSTRACT

The extraction by ethyl acetate and subsequent electrochemical detection of organosulfur containing molecules from garlic is demonstrated. The electrochemical results first evidence the high sensitivity of the process towards the model compound propyl disulfide. Through the in-situ formation of bromine at a platinum electrode the propyl disulfide can be readily detected at concentrations as low as 12.5 μM . Second, the work focuses on the detection of organosulfur from fresh garlic samples. Extraction of the organosulfur 'flavour' molecules is achieved with ethyl acetate. Addition of this extract to the electrochemical cell results in an analytically useful signal allowing the voltammetric peak height to be successfully correlated with the garlic strength, as measured using an organoleptic tasting panel.

KEYWORDS

Electrochemistry, Voltammetry, Bromine, Thiosulfinate, Sulfoxide

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29 1. Introduction

30 One of the most distinctive features of the genus *Allium* is the plants use of organosulfurs as
31 defensive secondary metabolites.(Block, 2010) In the cell, sulfoxide precursors are stored within the
32 cytoplasm and the enzyme alliinase is compartmentalised within the vacuole.(Lancaster & Collin,
33 1981) On cell damage, the sulfoxides are converted by the released alliinase to thiosulfinates
34 (organosulfur structures are shown in Figure 1). The main thiosulfinate formed by garlic (*A. Sativum*)
35 is allicin and this chemical species has been shown to stimulate the nociceptor TRPA1, hence, the
36 perceived pungency of fresh garlic. (Macpherson, Geierstanger, Viswanath, Bandell, Eid, Hwang, et
37 al., 2005) However, allicin is a reactive species and undergoes a number of different transformations
38 leading to a host of different possible poly- and disulfides.(Block, 2010) Consequently, garlic 'flavour'
39 relates to a range of different organosulfurs produced from the precursor sulfoxides. The sulfoxides
40 generally constitute between 2-25 g kg⁻¹ of the fresh weight of the bulb.(Horníčková, Kubec, Cejpek,
41 Velíšek, Ovesná, & Stavelikova, 2010) It is the concentration of these sulfoxides that determine the
42 levels of the thiosulfinates present after preparation of the garlic and hence they ultimately determine
43 the 'strength' of the garlic in terms of its flavour. However, the concentration of these sulfoxides is
44 found to be significantly influenced by the sub-species, harvest time and storage
45 conditions.(Horníčková, Kubec, Velíšek, Cejpek, Ovesná, & Stavelikova, 2011) For instance the
46 concentration of the sulfoxides has been previously found to increase by ~30% over the first eight
47 weeks of storage.(Horníčková, Kubec, Cejpek, Velíšek, Ovesná, & Stavelikova, 2010) Although
48 numerous methods are available for the quantification of the concentration of the sulfoxides, most
49 importantly HPLC,(Ziegler & Sticher, 1989) mass-spectrometry(Lee, Kim, & Lee, 2003) and capillary
50 electrophoresis,(Kubec & Dadáková, 2008) none of these methods are easily adaptable for being
51 undertaken by non-scientifically skilled personnel, for the regular determination of batch-to-batch
52 variability.

53 In the food industry the variation in garlic strength is routinely investigated by organoleptic tasting
54 panels. For these panels macerated garlic is diluted in sour cream(Toebe, Hoojjat, Hernandez, Giacin,
55 & Harte, 1990) and the panel asked to rank the strength of the garlic on a scale of 0-8. Although, this
56 yields some information regarding the strength of the garlic, the procedure is clearly liable to errors
57 due to the subjectivity of the measurement. Previous work by Compton *et al.* demonstrated how the
58 strength of macerated garlic may be objectively quantified via electrochemical bromination of
59 organosulfur.(Martindale, Aldous, Rees, & Compton, 2011) The reactivity of bromine towards
60 organosulfurs had long been recognised, where under non-aqueous conditions the reaction of thiols
61 with bromine is used for the near quantitative formation of the associated disulfide.(Wu, Rieke, &

62 Zhu, 1996) However, under aqueous conditions the oxidation can go further, leading ultimately to the
 63 sulfonate.(Oae & Doi, 1991; Young, 1937) The equations, presented in Figure 1, outline the
 64 stoichiometry for the reaction of bromine with both thiols and disulfides.
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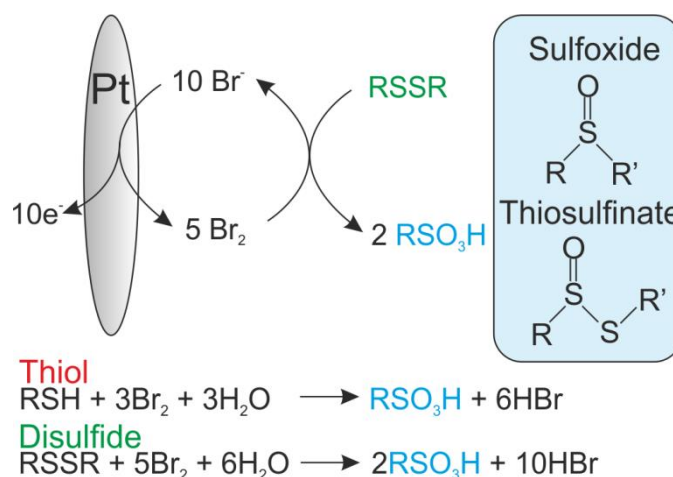


Figure 1: Schematic showing the electrocatalytic cycle used for the detection of organosulfurs. The bromide/bromine redox system acts as the homogeneous redox mediator, which is oxidized to bromine at the platinum electrode (Pt). Hence, the recorded electrochemical signal is proportional to the organosulfur concentration. The stoichiometry for the reactions shown corresponds to that found in the literature for the complete oxidation of thiol and disulfide species in an aqueous system by bromide.(Oae & Doi, 1991) The inset blue box depicts the chemical structures of the flavour pre-cursor sulfoxide and the main 'fresh' flavour molecule thiosulfinate.

66
 67 In the electrochemical experiment the organosulfur compounds are extracted from the raw garlic and
 68 added to the electrochemical cell. The electrochemical cell contains bromide which is oxidised to
 69 bromine at the electrode. This formed bromine reacts with the organosulfur leading to the
 70 reformation of the bromide ions. These bromide ions may then be re-oxidised at the electrode surface
 71 to bromine. This catalytic cycle (also shown in Figure 1), which results in the bromide being
 72 repeatedly oxidised, leads to an enhancement of the recorded electrochemical current. This increase
 73 in the current is directly related to the concentration of the organosulfur compounds present within
 74 solution and hence can be directly correlated to the strength of the garlic.

75 This work builds on the previous paper(Martindale, Aldous, Rees, & Compton, 2011) and through
 76 optimisation of the electrochemical conditions in terms of bromide concentration and electrode
 77 material, the sensitivity of the technique is enhanced, such that 12.5 μM of the model compound
 78 propyl disulfide is experimentally readily detectable. Second, the previous work is hindered by the
 79 use of relatively large quantities of acetonitrile (methyl cyanide) as the solvent used for the
 80 organosulfur extraction. This work utilises the significantly less toxic solvent ethyl acetate. Finally,
 81 having optimised the procedure the electrochemical quantification of the strength of six different

garlic samples (comprising of four different sub-species) is evidenced and a strong correlation ($R^2 = 0.974$) between the electrochemical and independent organoleptic tests is demonstrated, validating the developed methodology for the rapid quantification of the strength of garlic.

2. Experimental

All chemicals were purchased at the highest grade available and used directly without any further purification. Propyl disulfide (98%) and NaClO_4 (ACS Reagent, 98%) were sourced from Sigma Aldrich, UK; NaBr was sourced from May and Baker Ltd, UK. All solutions were prepared with ethyl acetate (HPLC Gradient grade, Sigma-Aldrich >99.7%) and deionised water of resistivity not less than $18.2 \text{ M}\Omega \text{ cm}$ at 298 K (Millipore UHQ, Vivendi, UK).

Garlic samples were provided by Beacon Foods Ltd (Brecon Powys, UK), and consisted of 6 samples; Chinese, Fresh White, Red Morado and three samples of Spanish Morado. The garlic cloves were peeled and chopped (macerated) in Beacon Foods Ltd kitchens then posted under refrigerated conditions. Extraction of the organosulfur compounds from the raw macerated garlic samples was achieved by addition of a given mass of garlic (0.1-1.0 g) to 2 mL of ethyl acetate contained in a centrifuge tube. The resulting mixture was subsequently mixed on a Vortex (Whirlimixer, Fisons Scientific Loughbough) for 1 minute, and then separated by centrifugation (Centrifuge 5702, Eppendorf, Hamburg, Germany) at 3000 relative centrifugal force (rcf) for 6 minutes. The resulting ethyl acetate supernatant containing the garlic organosulfur extract was added to the electrochemical cell.

All voltammetric measurements were recorded using an Autolab 101 computer-controlled potentiostat (Metrohm, Utrecht, The Netherlands). Experiments were performed using a three-electrode set-up, with a graphite rod and a Saturated Calomel Electrode (SCE +0.244 V vs SHE, BASi inc, Japan) as counter and reference, respectively. An in-house produced platinum macro-electrode (radius: 1.0 mm) was used as the working electrode, the electrode was prepared by securing the material inside an insulating PTFE surround to leave an exposed circular geometric surface. Renewal of the platinum surface was achieved by polishing with alumina slurries (1.0–0.3 μm , Buehler Ltd., USA). A beaker containing a total of 25 mL of electrolyte was employed and thermostated using a water bath at $25.0 \pm 0.3^\circ\text{C}$.

Sensory analysis of the garlic samples was performed by Beacon Foods (Brecon, UK), who also supplied the garlic samples. Organoleptic testing was undertaken by mixing one gram of the raw garlic with 99 g of sour cream. A panel of ten untrained taste testers were then asked to rate the strength of the garlic/sour cream mix on a scale of zero to eight (where zero represented no flavor).

115 From these results the mean and error of the mean were calculated.

116 3. Results and Discussion

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118 In the following work, first, the electrochemical detection of a model disulfide is evidenced (Section
119 3.1), followed by the use of real samples where the garlic organosulfur species are extracted using
120 ethyl acetate (Section 3.2). Finally (Section 3.3), a strong correlation between the organoleptic and
121 electrochemical methodologies is evidenced.

122 3.1 Propyl disulfide detection

123 Figure 2 shows, the oxidation of 1.875 mM sodium bromide (electrolyte; 0.1 M NaClO₄ and 5% by
124 volume ethyl acetate) recorded at a platinum macro-electrode, the voltammetric scan is started at 0.0
125 V (vs SCE) and swept anodically to +1.2 V, the electrode potential was subsequently scanned back to
126 0.0 V to complete the cyclic voltammogram (scan rate = 100 mV s⁻¹). On the forward sweep a large
127 oxidative peak is observed at (0.97 V) corresponding to the one-electron oxidation of the bromide ions
128 to bromine. On the reverse scan the corresponding reduction wave is observed at +0.88 V. The
129 relatively small peak-to-peak separation (88 mV) for the system (as compared to previous results on
130 carbon) is due to the use of a platinum electrode, where the interfacial electron transfer kinetics are
131 significantly faster. It should be commented that at high bromide concentrations the electrochemical
132 formation of bromine has been reported to be self-inhibiting with the associated formation of
133 tribromide (Br₃⁻). (Allen, Buzzeo, Villagrán, Hardacre, & Compton, 2005) This self-inhibition leads

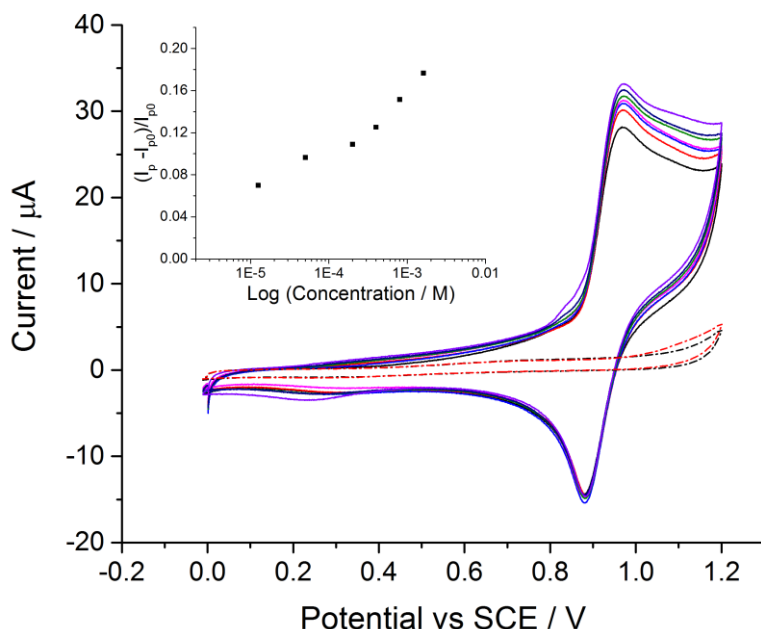


Figure 2: The voltammetric oxidation of 1.875 mM Br⁻ on a platinum macroelectrode (radius = 1.0 mm) in the presence of increasing concentrations of propyl disulfide (black: 0 mM, red: 0.0125 mM, blue: 0.05 mM, pink: 0.2 mM, green: 0.4 mM, dark blue: 0.8 mM and purple: 1.6 mM). Also depicted is the voltammetric response in the absence of the bromide with (1 mM, red dashed line) and without (black dashed line), the presence of 1.0 mM disulfide. The inlay depicts the recorded normalised peak current versus the log of the disulfide concentration, where i_{p0} is the oxidative peak height in the absence of propyl disulfide.

both to a minimisation of the forward wave and an associated decrease in the reductive peak height. On addition of propyl disulfide to the solution the oxidative voltammetric wave is observed to increase. Conversely, the dashed red line in Figure 2 shows the voltammetric response of the platinum electrode to the presence of 1 mM propyl disulfide but in the absence of bromide; no clear oxidative wave is observed with the oxidation being partially distorted by the onset of the breakdown of solvent. The inlay of Figure 2 depicts the normalised peak current versus the solution phase concentration of the propyl disulfide. On the normalised peak current scale a value of zero represents a peak height that is equal to that found for the oxidation of bromide in the absence of the organosulfur. Clearly from the presented data detection of the propyl disulfide even at the concentration of 12.5 μ M is readily attainable. This sensitivity towards the presence of the disulfide partially reflects the stoichiometry of the bromination reaction as given in shown in Figure 1; consequently, for each disulfide molecule oxidised up to 10 electrons may be transferred.

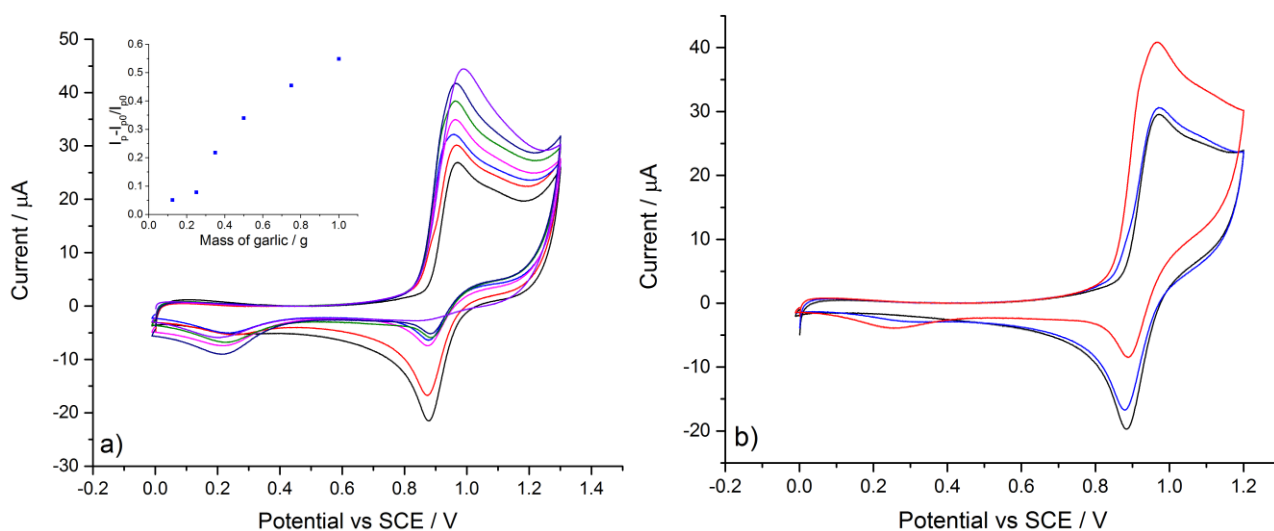


Figure 3: a) The oxidation of bromide in the presence of ethyl acetate containing garlic (Spanish Morado) extract (electrochemical cell total ethyl acetate is 5% by volume), the mass of garlic used for the extraction procedure has been varied between 0.0-1.0 g (black: 0 g, red: 0.125 g, blue: 0.25 g, pink: 0.35 g, green: 0.5 g, dark blue: 0.75g and purple: 1.0 g). The inlay depicts the recorded normalized peak current against the mass of garlic used in the extraction procedure. b) The oxidation of bromide in the presence of ethyl acetate containing garlic extract from two different sub-species used in the extraction procedure, black: control- no garlic, blue: 0.5 g of Chinese garlic and red: 0.5 g of Spanish Morado.

3.2 Electrochemical quantification of garlic samples

Having evidenced the sensitivity of the electrochemical bromide/bromine system towards disulfides the article next turns to the detection of organosulfur in raw macerated garlic samples. For the extraction procedure the garlic sample was added to 2 mL of ethyl acetate, the sample was subsequently mixed on a vortex mixer for one minute. Separation of the garlic solids from the ethyl acetate was achieved by 6 minutes centrifugation (3,000 rcf). An aliquot of the ethyl acetate

supernatant - now containing the organosulfur compounds - was subsequently added to the electrochemical cell. Note that ethyl acetate is only miscible with water up to approximately 8%,(Altshuller & Everson, 1953) consequently the relative volume of the extract that can be added to the cell is limited. Figure 3 depicts the voltammetric response for the oxidation of the bromide in the presence of the ethyl acetate extract. The mass of garlic used during the extraction process was varied from 0.0 to 1.0 g (as mixed with the 2 mL of ethyl acetate). Clearly, from Figure 3 a), as the quantity of garlic used for the extraction procedure is increased the magnitude of the oxidative wave shows a corresponding increase, the inlay depicts the normalized peak current as a function of the garlic mass used within the extraction procedure. Figure 3 b) demonstrates the voltammetric response for the extraction of the organosulfur from two differing garlic species; Chinese and Spanish Morado (0.5 g was used for both extractions). The increase in the voltammetric peak is observed to be significantly greater for the Spanish Morado garlic extract. This result is fully consistent with literature reported values for the total sulfoxide contents of these two sub-species.(Huchette, Kahane, Auger, Arnault, & Bellamy; Kubec, Svobodová, & Velíšek, 1999) Although it is again highlighted that a plant's sulfoxide concentration is dependent upon a number of factors, the Chinese garlic has been reported to contain 5.3 g kg⁻¹ sulfoxides(Kubec, Svobodová, & Velíšek, 1999) as compared to Spanish Morado which contains 19.9 g kg⁻¹. This latter number has been estimated from a reported alliin content of Spanish Morado (90 nmol mg⁻¹) (Huchette, Kahane, Auger, Arnault, & Bellamy) and scaled accordingly, assuming that alliin constitutes ~80% of the total sulfoxide concentration. With the range of reported values for the total sulfoxide concentration generally ranging from 2 to 25 g kg⁻¹,(Horníčková, Kubec, Cejpek, Velíšek, Ovesná, & Stavelikova, 2010) then the above literature values for the Chinese and Spanish Morado garlic is consistent with the varieties having 'weak' and 'medium/strong' flavors respectively. It is commented that the bromination method employed may also lead to enhancement of the catalytic current through reaction of the electrochemical formed bromine with other sulfur and nitrogen containing species present within the ethyl acetate extract (thiamine for example). However, importantly it has been previously shown that the oxidation of amines does not occur to any significant extent over the voltammetric timescale.(Valero-Ruiz, González-Sánchez, Batchelor-McAuley, & Compton, 2016) This lower reactivity of the bromine towards amines and the relatively high concentrations of the sulfoxides present within the garlic ensures that the error associated with these side reactions is minimized.

3.3 Organoleptic versus electrochemical studies

Finally, this work turns to quantify the strength of six different garlic samples, comprising of four

187 different sub-species; Chinese, Fresh White, Red Morado, and Spanish Morado (three different
 188 batches). The strength of the garlic flavor is studied via both the developed electrochemical technique
 189 and an independent organoleptic taste testing. For the electrochemical procedure, for each sample
 190 three separate extractions were undertaken using 0.5 g of macerated garlic each time. The extraction
 191 used the minced garlic as supplied and was not processed further prior to addition of the ethyl
 192 acetate. For each extract three repeat voltammograms were performed, allowing the variability

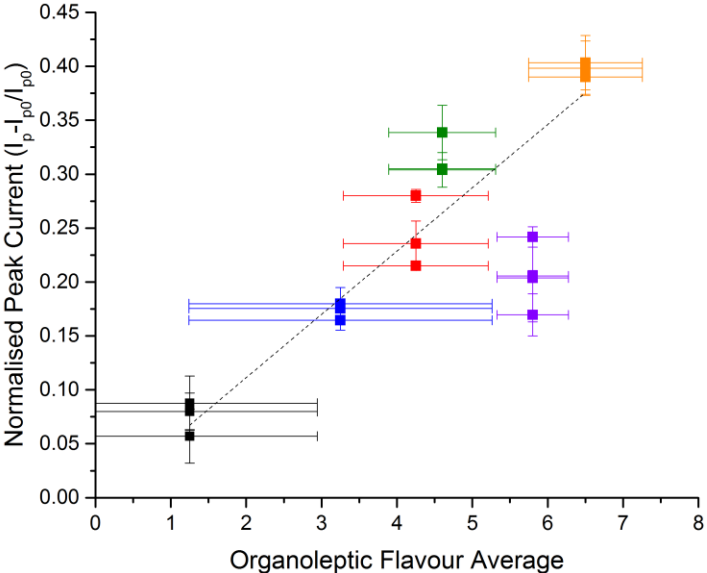


Figure 4: Plot of the electrochemically measured garlic strength (normalized peak current) versus the independent organoleptic results for 6 different garlic samples. Black: Chinese, Blue: Fresh White, Red: Red Morado and, Purple, Green and Orange: three different batches of Spanish Morado garlic (Batches 1, 2 and 3). Dotted line shows the line of best fit for between the two datasets showing a strong correlation ($R^2 = 0.974$). Error bars for organoleptic flavor testing represent the error of the mean and not the standard deviation- further discussion is provided in the text.

193 associated with the electrochemical procedure itself to be evidenced. The organoleptic taste tests
 194 were performed by a panel consisting of ten members. 1 g of the macerated garlic was added to 99
 195 grams of sour cream and the panel asked to rate the strength of the flavor on a scale of 0-8, where
 196 zero represents no flavor and 8 is a strong flavor. From this organoleptic test the average value and
 197 the standard error of the mean were calculated. Note the standard error is used (i.e. the standard
 198 deviation/ $N^{0.5}$, where N is the number of testers) and not the standard deviation as in the current
 199 work we are interested in the strength of the garlic and not the variation in human perception of the
 200 strength of flavor. As an alternate comparison other analytical techniques such as HPLC or capillary
 201 electrophoresis may provide further information regarding the quantification of the strength of the
 202 garlic samples. However it is commented; first, due to the fact that taste of garlic arises from a variety
 203 of different organosulfur species any alternate detection techniques require validation against
 204 organoleptic data. Second, organoleptic taste panels are regularly used as an industrial standard for
 205 determining the strength of a garlic sample.

Figure 4 plots the electrochemically measured garlic strengths for each sample (with three repeat extractions for each sample) against the organoleptic measurement. As can be seen a strong correlation between the two methodologies is evidenced where the line of best fit has an R^2 value of 0.974. It is worthwhile commenting as follows. First, the errors on the organoleptic data are relatively large. Second, the three Spanish Morado samples exhibited a distinct variation in their recorded strength, and the results for batch 1 (purple) appear off trend. Third, for each extraction the error associated with the electrochemical detection methodology are comparatively and favorably small. However, some variation is observed between the individual extractions. This variation is not large enough to explain the anomalous behavior of the Batch 1 Spanish Morado (Purple Figure 4). In terms of the variability of the electrochemical results obtained for different extractions two main possibilities arise either the variability occurs due to variations in the extraction efficiency and/or the bulk sample from which the 0.5 g of garlic was taken is significantly heterogeneous. We note that the heterogeneity of the garlic sample would not be observable in the organoleptic data as only one 1 g sample was used per batch tested. Moreover, during the course of the electrochemical experiments it was noted that good mixing of the bulk garlic (1 kg tub) prior to removal of the 0.5 g aliquot to be used for the extraction was imperative to ensure more reproducible results. Consequently, on the basis of this we speculate that further maceration/processing of the raw garlic sample prior to the extraction procedure may likely improve the reproducibility of the results for each extraction. However, even in light of these noted variations the developed electrochemical technique compares exceptionally favorably with the organoleptic testing of the garlic samples, while at the same time, relying on non-quantifiable and subjective flavor impressions can be avoided.

4. Conclusions

The use of organosulfur bromination for the electrochemical detection of the strength of garlic has been successfully verified against independent organoleptic testing. The sensitivity of the electrochemical methodology has been improved through the use of a platinum macroelectrode, such that, the detection of disulfide at the concentration of tens of micromolar is readily attainable. Extraction of the garlic based organosulfur compounds is achieved via a simple extraction methodology using the organic solvent ethyl acetate. Hence, addition of the garlic extract to the electrochemical cell yields a readily measurable change in the magnitude of the voltammetric peak height. The peak is shown to be larger in the presence of higher concentrations of organosulfur, leading to a simple methodology by which the strength of a garlic sample may be objectively

239 determined, demonstrating the applicability of the methodology for use by non-scientifically skilled
240 personnel.

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249

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