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Talanta 64 (2004) 894–898
Talanta
www.elsevier.com/locate/talanta

Determination of proline in wine using flow injection analysis with tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence detection

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Received 27 January 2004; received in revised form 26 March 2004; accepted 26 March 2004
Available online 24 June 2004

Abstract

Flow injection methodology is described for the determination of proline in red and white wines using tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence detection. Selective conditions were achieved for proline at pH 10, while other amino acids and wine components did not interfere. The precision of the method was less than 1.00% (R.S.D.) for five replicates of a standard (4×10^{-6} M) and the detection limit was 1×10^{-8} M. The level of proline in white and sparkling wines using the developed methodology was equivalent to those achieved using HPLC-FMOC amino acid analysis. SPE removal of phenolic material was required for red wines to minimize $Ru(bipy)_3^{3+}$ consumption and its associated effect on accuracy.

Keywords: Tris(2,2′-bipyridyl)ruthenium(II); Chemiluminescence; $Ru(bipy)_3^{3+}$; Flow injection analysis; Proline; Wine

1. Introduction

The nitrogen levels of must and wines is relatively small ranging from 0.06 to 2.4 g l^{-1} and is comprised of amino acids, peptides, proteins, amines and peptones [1,2]. These nitrogenous species play a vital role in winemaking maintaining the correct yeast activity [3]. The concentration and type of nitrogenous compounds present can affect the rate of fermentation and the bouquet of the resultant wine [2]. Slow or incomplete fermentations and hydrogen sulfide production may result from insufficient levels of free assimilable nitrogen [1].

The amino acid content and composition of the berry is influenced by a variety of factors including fruit maturity and cultivar [4]. Proline is the predominant amino acid in the grape berry (up to 2 g l^{-1}) and accounts for 30–80% of the total nitrogen content [3], thus several analytical methodologies have been developed for its determination within the wine and food industry [3]. The level of proline has been related to the total nitrogen content, cultivar type and wine quality. The most commonly employed methods for the determination of amino acids in wine using HPLC have been reviewed by Lehtonen [5]. These chromatographic approaches may require pre-concentration followed by derivatisation and take more than 30 min per analytical cycle. The direct determination of proline following derivatisation with ninhydrin has been reported by a number of workers [6,7]. The method described by Ough et al. [7] quantified free proline in grapes and wine, however lysine, hydroxyproline, tryptophan and glutamine may have interfered.

Chemiluminescence with flow analysis [8] has been used to directly determine a range of analytes in complex matrices due to the selective conditions achievable with certain reactions [9–11]. Proline has been previously determined using tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence [9,12–16] and the analytical figures of merit from those investigations has been summarized in Table 1. A recent publication from our laboratory [9] reported the selective chemiluminescence detection of proline in an equimolar mixture of the 20 naturally occurring amino acids. The present investigation extends this work [9] to the determination of proline in various wines using flow injection analysis (FIA) with tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence detection.

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doi:10.1016/j.talanta.2004.03.065

Table 1
The determination of proline using $Ru(bipy)_3^{3+}$ chemiluminescence

$Ru(bipy)_3^{3+}$ generation	Comments	Matrix	Limit of detection (M)	Reference
External electrochemical	FIA, LC study conducted at pH 5.8 on 21 amino acids	Synthetic collagen	1×10^{-6}	[12]
External electrochemical	FIA, 50 mM H_3BO_3 pH 10	Synthetic	Not reported	[13]
External electrochemical	FIA, relative intensities for 15 amino acids, LC separation of Gramicidin D digest	Protein digest	Not reported	[14]
In-situ electrochemical/ glassy carbon	FIA, LC, boric acid/sodium acetate pH 10	Synthetic	5×10^{-9}	[15]
In-situ and external electrochemical	FIA, various modes of $Ru(bipy)_3^{3+}$ generation	Synthetic	External 5×10^{-7} In situ 5×10^{-7} In situ immobilized 1×10^{-6}	[16]
Chemical oxidation	FIA, pH 10 sodium tetraborate	Equimolar amino acid mixture	4×10^{-9}	[9]

2. Experimental

2.1. Instrumentation

The flow injection analysis manifold, as shown in Fig. 1, was used throughout this study. A Gilson Minipuls 3 peristaltic pump (John Morris, Australia) fitted with bridged PVC tubing (1.02 mm i.d.) propelled both sample and reagent streams. All other tubing was 0.8 mm i.d. teflon (Protech, Australia) unless otherwise specified. The tris(2,2′-bipyridyl)ruthenium(III) solutions were injected manually using a six-port injection valve (Valco instruments, Texas) into a water carrier which then merged with the sample stream in close proximity to a coiled teflon flow cell mounted flush against the photomultiplier tube (H5783-01 Hamamatsu, Stantron). The flow cell, photomultiplier tube and voltage divider were enclosed in a light tight housing. Output from the photomultiplier tuber was monitored with a chart recorder (YEM Type 3066, Yokogawa Hokushin Electric, Tokyo) and responses were measured manually as peak height.

Amino acid determinations were also performed using a Hewlett-Packard 1100 series HPLC employing an injection volume of 10 μL onto a C18 Lunar column (250mm × 4.6 mm i.d., 5 μm, Phenomenex Australia) with a gradient elution (see Table 2) and absorption detection at 270 nm controlled by a Vectra X_m series 4 data analysis workstation.

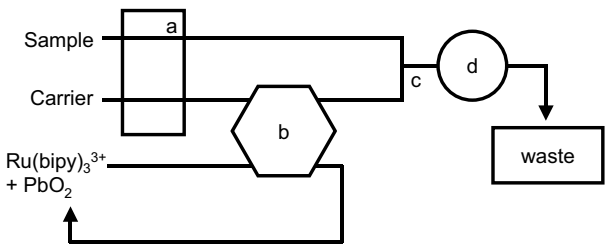

Fig. 1. Schematic of FIA manifold. Components: (a) peristaltic pump, (b) injection valve, (c) T-piece, (d) detector.

Dilution of standard and sample solutions were performed using a Gilson 402 dilutor dispensor (Melbourne, Australia), while pH measurements were performed using a Jenco electronics 6071 pH vison (CHK engineering, Australia).

2.2. Reagents and samples

All reagents were of analytical grade unless otherwise specified and solutions were prepared with deionised water (Millipore, MilliQ Water System, Bedford, MA). Stock solutions (1×10^{-3} M) of the L-amino acids: alanine, aspartic acid, glycine, histidine, isoleucine, lysine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine (Sigma–Aldrich, Castle Hill, NSW, Australia), asparagine monohydrate, glutamine, leucine, methionine (Calbiochem, La Jolla, CA, USA), glutamic acid (sodium salt), proline (BDH, Poole, Dorset, UK), arginine (Ajax (APS), Auburn, NSW, Australia) and cysteine (Hopkin & Williams, Chadwell Heath, Essex, UK), were prepared by dissolution of the solid in deionised water and used within three days. A synthetic wine matrix consisting of tartaric acid (7.3 g l^{-1}, Ajax, Auburn, NSW, Australia), sodium metabisulphite (25 ppm, Ajax), ethanol (12%, v/v, Merck, Australia) and glucose (8 g l^{-1}, BDH) was prepared and acidified with dilute hydrochloric acid to pH 3.3. The derivatisation reagent 9-fluorenylmethyl chloroformate, boric acid, and sodium tetraborate were purchased from Sigma–Aldrich.

Table 2
Chromatographic conditions for FMOC amino acid analysis

Time (min)	6 mM sodium phosphate pH 6.5 (%)	Acetonitrile (%)	Flow (ml min^{-1})
0	80	20	0.8
5	80	20	0.8
20	65	35	0.8
30	30	70	0.8
34.05	0	100	1
37	0	100	1
37.05	80	20	1
40	80	20	1

2.3. Procedures

The derivatisation of amino acids with 9-fluorenylmethyl chloroformate was performed according to the method described by Bauza et al. [17] with slight modifications. A 100 μL aliquot of either standard or wine sample (diluted 1 + 1) was mixed with borate buffer (100 μL, 200 mM boric acid pH 8.5). The 9-fluorenylmethyl chloroformate (200 μL, 8 mg mL^{-1} in tetrahydrofuran) was added and reacted for 3 min, followed by addition of cleavage solution (100 μL, 0.5 M NH_3) left to stand for a further 3 min. The reaction was quenched by the addition of a solution of tetrahydrofuran (BDH) and acetic acid (600 μL, 4 +1) and immediately analysed by HPLC.

Solutions of tris(2,2′-bipyridyl)ruthenium(III) (1 mM, Fluka) were prepared in 0.02 M sulfuric acid using a recirculating system described previously [18], followed by injection (70 μL) into a water carrier stream.

For flow injection analysis, wine samples were diluted by a factor of 1000 with sodium tetraborate (50 mM, pH 10) using an auto dilutor. Red wines were also subjected to solid phase extractions using Strata X tubes (30 mg/1 mL, Phenomenex). The tubes were initially conditioned with methanol followed by deionised water. The wine samples (200 μL) were then added and eluted using a 5% methanol solution.

A variety of wine styles including shiraz, cabernet sauvignon, chardonnay, traminer riesling and riesling were supplied by Stonehaven winery (B.R.L. Hardy, Australia). Four sparkling wines, two produced by mêthode champenoise, and two by transfer and carbonation were also analysed.

3. Results and discussion

3.1. Preliminary experiments

The relative chemiluminescence response of all of the 20 naturally occurring amino acids reacted with tris(2,2′-bipyridyl)ruthenium(III) is shown in Table 3. Under the conditions used, the magnitude of either positive or negative interference from other amino acids was less than 2% at the equivalent concentration. The response for histidine and tryptophan, which also contain a secondary amine, was found to be minimal which has also been observed by other workers at these conditions [15]. Tryptophan has been observed to elicit a strong chemiluminescence signal in acidic conditions however, the variation in response to pH are as yet unknown [19]. Interestingly, a standard solution containing all 20 amino acids (all at 5×10^{-6} M) only produced an enhancement of 0.6% over the signal for the 5×10^{-6} M proline standard.

An amino acid mixture that approximated the composition of a wine sample as described by Lehtonen was analysed for proline at three concentrations 4×10^{-5}, 4×10^{-6}, 4×10^{-7} M). Aqueous standards of proline at the same concentrations as added to the wine amino acid mixture [5] were also determined. As can be seen in Table 4 these results further underline the inherent selectivity of this chemiluminescence for the determination of proline.

Table 3
Relative chemiluminescence response of amino acids vs. proline

Amino acid	Relative response (%) $Ru(bipy)_3^{3+}$ CL 5×10^{-6} M
Proline	100.00
Arginine	−0.34
Tryptophan	0.61
Asparagine	−0.38
Cysteine	0.13
Glycine	−1.91
Phenylalanine	−0.42
Lysine	−0.39
Glutamic acid	0.69
Alanine	−1.53
Glutamine	−0.71
Leucine	0.38
Serine	−1.32
Histidine	−0.08
Valine	−1.42
Isoleucine	0.75
Threonine	−1.98
Tyrosine	0.43
Methionine	1.34
Aspartic acid	−0.54
Equimolar amino acid mix	100.6

(1 mM $Ru(bipy)_3^{3+}$, 70 μL, total flow rate 11.5 mL min^{-1}, pH 10).

Other classes of compounds including sugars, acids and phenolics that are present in wine [2] at substantially higher levels than proline may also enhance or quench the chemiluminescence response [8]. To examine the effect of these species, proline was added to a synthetic wine matrix [20] that was characteristic of the levels of these compounds. The relative responses of the individual components and synthetic wine are shown in Table 5. Tartaric acid was observed to elicit weak chemiluminescence which has been described by previous workers [21]. Quenching of chemiluminescence is generally observed for phenolic compounds, however, other investigations in alkaline conditions have produced responses greater than the blank which was also observed to occur with catechin [22,23].The overall enhancement from the synthetic wine matrix was +2.5% which represents an acceptable level of interference that could be compensated for by calibration with standard additions.

The response of eight aqueous standard solutions of proline (1×10^{-8} to 5×10^{-5} M) gave a calibration func-

Table 4
Determination of proline in wine amino acid standard

Concentration (M)	Proline standard (mV)	Synthetic wine a.a. mixture (mV)	Ratio
4.0×10^{-5}	321.6	316.6	98.4
4.0×10^{-6}	30.4	30.6	100.9
4.0×10^{-7}	5.1	5.2	100.8

(1 mM $Ru(bipy)_3^{3+}$, 70 μL, total flow rate 11.5 mL min^{-1}, pH 10).

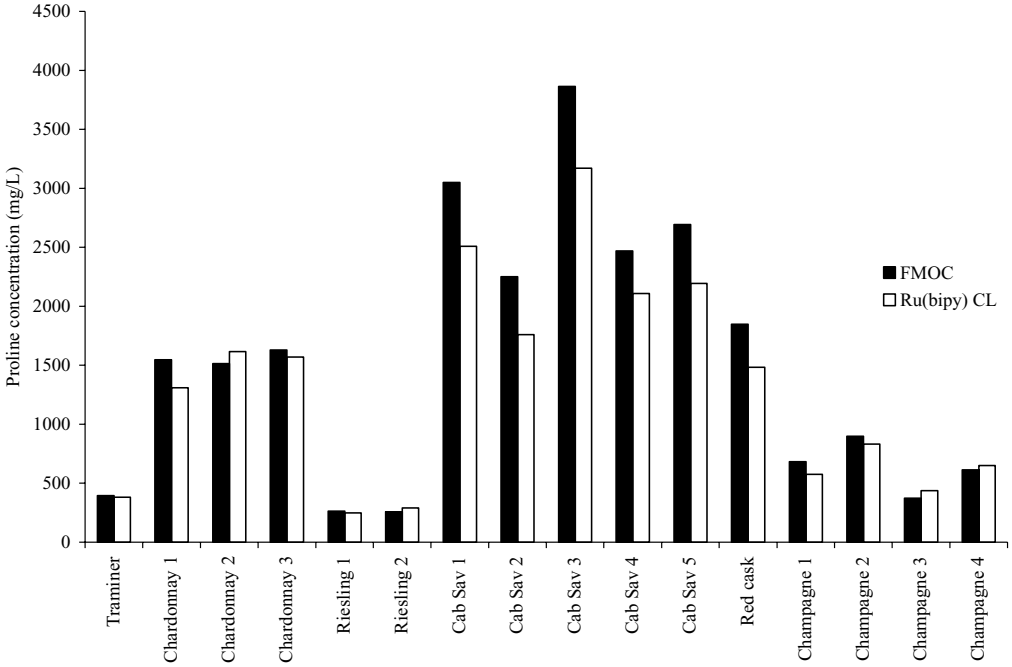

Fig. 2. Comparison of proline concentrations in various wine samples using $Ru(bipy)_3^{3+}$ chemiluminescence and FMOC amino acid analysis.

tion of $y = 1.09 \times 10^7 x + 0.99$ ($R^2 = 0.9999$). Where y = chemiluminescence response in mV and x = proline concentration in mol l^{-1}. The detection limit was calculated from three times the standard deviation of the blank signal to be 1×10^{-8} M, which compares favourably to those reported previously with tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence [9,12–16].

3.2. Determination of proline in wine

Sixteen wine samples (four sparkling, six white and six reds) were analysed for proline using both FIA and HPLC and these results are summarised in Fig. 2. For white and sparkling wines, the correlation between the two methodologies was excellent with only minor random differences,

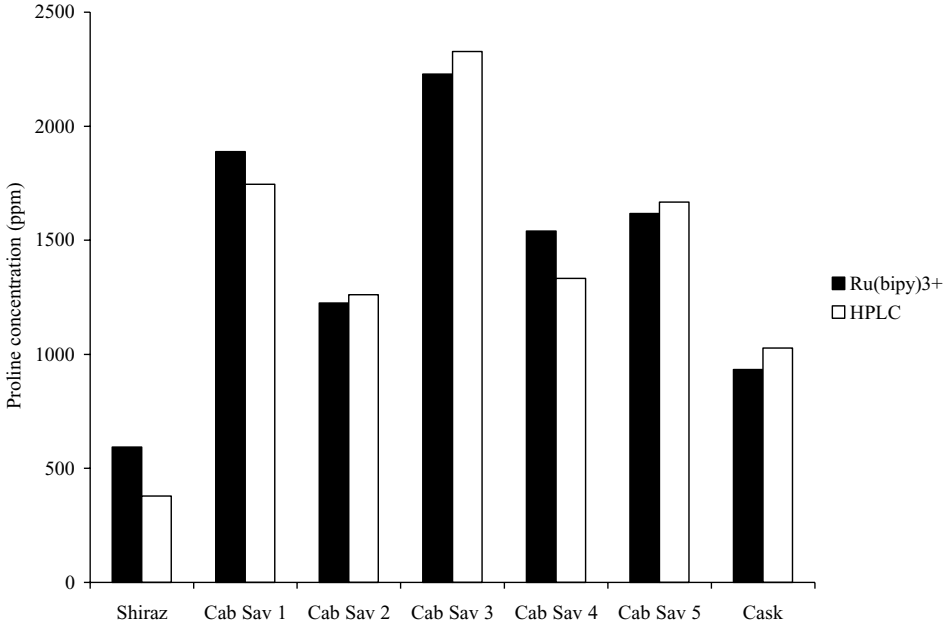

Fig. 3. Comparison of proline concentrations in red wine using $Ru(bipy)_3^{3+}$ with SPE and FMOC amino acid analysis.

Table 5
Relative chemiluminescence response from possible interferents present in wine

Sample	Concentration ($g l^{-1}$)	Relative response to proline
Proline	0.92	100.00
Ethanol	120	−0.39
Tartaric acid	7.3	2.22
Sulphite	0.03	−0.39
Glucose	8.0	−0.92
Catechin	1.0	5.88
Caffeic acid	0.14	−3.92
Total		102.50

(1 mM $Ru(bipy)_3^{3+}$, 70 μL, total flow rate 11.5 mL min^{-1}, pH 10).

possibly attributable to errors associated with pipetting low volumes (100 μL) during the FMOC derivatisation procedure. The high dilution factors used for the FIA determinations appeared to have ameliorated the affects of the concomitant interferents. The proline content of the white and sparkling wines varied between 270 and 1640 mg l^{-1} which is similar to values previously reported [5]. The relatively large range of proline concentrations is consistent with different grape varieties and enological practices [2]. As expected [7], the six red wines contained higher levels of proline than the white and sparkling varieties. However the FIA results were, uncharacteristically, lower (approximately 80%) than those achieved using HPLC. The reduced response may have resulted from either quenching of the chemiluminescence or consumption of the reagent via non-light-emitting mechanisms [8]. As red wine contains relatively high levels of easily oxidisable phenolic compounds [2] these were considered to be the likely candidates for lowering the effective reagent concentration. Removal of these components was carried out using solid phase extraction, using a non-polar stationary phase, which eluted the fraction of interest using a 5% methanol solution, while retaining the red pigments and other phenolic compounds. Breakthrough of the anthocyanins was observed to occur above 250 μL although this amount may vary according to the phenolic levels of the wine. Following collection and dilution of the eluent the level of proline was re-determined by FIA and found to be in closer agreement (±10%) to that achieved with HPLC (see Fig. 3). Larger R.S.D. are observed with SPE and the FMOC amino acid analysis due to manual pipetting of small volumes (200 μL or less).

4. Conclusions

Tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence and FIA have been used to provide a simple and robust method for the accurate determination of proline in a number of wine styles. The major advantage of this approach, compared to established methods [5], is the analytical throughput (up to 60 h^{-1} for white and 20 h^{-1} for red wine) which includes sample preparation; with only aqueous dilution required for white and sparkling wines and the removal of phenolics by simple SPE for red wines which can be undertaken for multiple samples in parallel. The rapid determination of proline at the winery would provide the winemaker with information regarding the available nitrogen levels and grape cultivar and maturity.

Acknowledgements

The authors would like to thank B.R.L. Hardy for the provision of the wine samples and Phenomenex for the SPE catridges. The work was supported by the Centre for Chiral and Molecular Technologies, Deakin University and an Australian Postgraduate Award (J.W.C).

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