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Short communication

Determination of carnosine in feed and meat by high-performance anion-exchange chromatography with integrated pulsed amperometric detection

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Abstract

Carnosine (β-alanyl-L-histidine) is a dipeptide regarded as an important molecular marker of the presence of processed animal proteins including meat and bone meal in animal feed. For its identification and quantification a sensitive and selective method by high-performance anion-exchange chromatography coupled with integrated pulsed amperometric detection (HPAEC–IPAD) was developed. The assay is based on isocratic elution with 100 mM NaOH as the mobile phase. Interferences of real matrices were efficiently removed; carnosine could be determined at the concentration ranges 0.1–100 μM with a rather low detection limit of 0.23 ng. Unlike feeds for dogs and cats, no carnosine peak was observed in all examined feeds for ruminants. The good analytical characteristics allowed carnosine determination down to 5 μg/g of feed.

Keywords: Food analysis; Pulsed amperometric detection; Carnosine

1. Introduction

L-Carnosine or β-alanyl-L-histidine (Fig. 1) is one of the most abundant nitrogenous compounds occurring in the nonproteic fraction of vertebrate skeletal, cardiac, and nervous tissues at concentrations up to 50 mM [1,2]. Since its discovery in beef extracts at the beginning of the century by Gulewitsch and Amiradzibi [3], carnosine has been also found in brain, olfactory epithelium, bulbs [4], and crystalline lens [5,6]. Although several roles of carnosine have been postulated, its precise functions in biological systems are not fully clarified. It has been suggested to serve as a buffer in muscle to offset the production of lactic acid during exercise [7,8] and as neurotransmitter in brain [9]; carnosine has excellent potential to act as a natural antioxidant with hydroxyl-radical- and singlet oxygen-scavenging and lipid peroxidase activities [10–13], thus, it may be useful to prevent, or partially reverse, lens cataracts [5,13].

As carnosine belongs exclusively to animal world, its occurrence in feeds clearly indicates presence of products of animal origin in feed. The ban on using processed animal proteins as feed additive for all farmed animals is an important measure to prevent the spread of transmissible spongiform encephalopathies (TSE); the official method to prove animal-originated adulteration, according to the European legislation [14], is the microscope examination [15]. Nevertheless, being the microscopic technique time-consuming and requiring expensive equipment and experienced operators, new analytical methods for feeds analysis are in development. Recently, Schönherr [16] proposed a chromatographic method for carnosine determination in animal feed as a possible alternative, which is based on the fluorescent detection upon derivatization with carbazole-9-carbonyl chloride. The separation is performed by reverse phase liquid chromatography using a C18 column and a binary gradient elution with an acetate buffer and a solution of acetonitrile, methanol, and tetrahydrofuran. The reason why fluorescent adducts of carnosine are preferred is due to its low UV absorbance. Indeed, several liquid chromatographic methods for determination of carnosine, and related imidazole dipeptides, in different matrices are based on a pre-column derivatization

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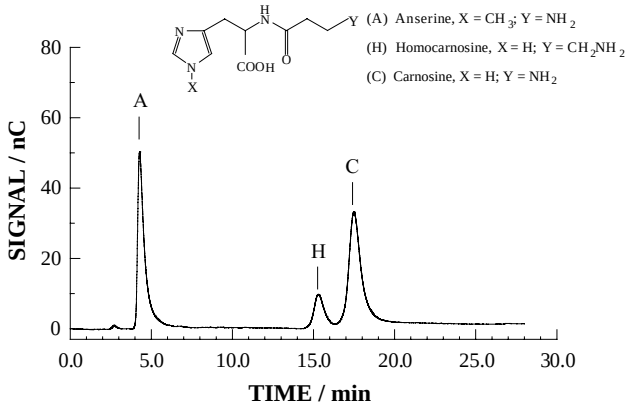

Fig. 1. Separation in HPAEC with integrated pulsed amperometric detection of a mixed standard solution containing: 3 μM anserine (A), 10 μM homocarnosine (H), 10 μM carnosine (C). Eluent, 100 mM NaOH at a flow rate of 0.15 ml/min. Column, Dionex AminoPac PA10 plus guard column. Column temperature, 25 °C. Volume injected, 10 μl. The potential waveform, detailed in Table 2, was employed at a gold working electrode.

coupled to reversed phase or ion exchange separations [17–22].

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD) has demonstrated to be very effective for the determination of underivatized carbohydrates and amino acids at nanomolar levels in complex mixtures [23–28]. Here, we describe the development and validation of an high-performance anion-exchange chromatography coupled with integrated pulsed amperometric detection (HPAEC–IPAD) assay for the quantitation of carnosine without recurring to derivatization. The proposed method, combined to a simple clean-up procedure, will be demonstrated useful and reliable to ascertain the presence of products of animal origin in feed samples.

2. Materials and methods

2.1. Chemicals

Chemical standards of L-carnosine approximately 99%, L-anserine nitrate salt (β-alanyl-1-methyl-L-histidine) >99%, and L-homocarnosine (γ-aminobutyryl-L-histidine) 99% were purchased from Sigma-Aldrich (Steinheim, Germany); methanol, carbonate-free sodium hydroxide (50% (w/w)), sodium acetate, sodium borate, phosphoric acid were also from Sigma-Aldrich. All the reagents used in this study were of the highest purity available. Stock solutions were prepared with pure water supplied by Milli-Q RG unit from Millipore (Bedford, MA, USA). Sodium hydroxide solutions used as eluents were prepared by dilution of a carbonate-free 50% (w/w) NaOH solution in water, previously filtered with a 0.45 μm membrane and degassed with nitrogen.

2.2. Apparatus and method

Chromatographic separations were performed on a Dionex apparatus (Sunnyvale, CA, USA) equipped with a metal-free isocratic pump (Model IP 20), a Rheodyne injection valve (model RH9125, Cotati, CA, USA) with a 10 μl loop and a pulsed amperometric detector (model ED40). The flow-through detection cell is made from a 1.0-mm diameter gold working electrode and a standard combination pH-Ag|AgCl reference electrode using the half-cell Ag|AgCl as the reference electrode; the titanium body of the cell served as the counter electrode. The active volume of the cell was approximately 0.2 μl. All the separations were performed using the following Dionex columns, coupled with the corresponding guard columns: microbore CarboPAC PA1 (250 mm × 2 mm i.d.) and AminoPAC PA10 (250 mm × 2 mm i.d.); the flow rates, applied during the experiments, were described in the figure legends. The column temperature (15–35 °C) was controlled using a homemade water jacket coupled with a circulating water bath model WK4DS from Colora (Colora, Messtechnik GmbH, Germany). The separations were performed by isocratic elution with NaOH at concentrations ranging from 80 to 150 mM, with eventually addition of 10 mM sodium acetate. After sparging with N_2 for 15 min, the reservoir bottles (DX 500 2 l bottles, Dionex) were closed and pressurized with pure nitrogen to 0.8 MPa. Data acquisition and processing was performed using the PeakNet Chromatography Workstation software, version 5.1.

2.3. Standard and working solutions

Stock solutions of 10 mM of carnosine, anserine and homocarnosine were prepared in water and stored at 4 °C. Working standard solutions were prepared on the day of use by dilution with the mobile phase. The carnosine contents in meat samples were obtained by standard additions.

2.4. Samples and sample preparation

Bovine and ovine feeds were kindly supplied by local breeders. Additional samples of feeds for adult cats and dogs as well as lyophilized meat samples were bought in local markets. The sample preparation was that described by Schönherr [16] and slightly modified. Briefly, 5 g of feed samples were suspended in 100 ml of water and mixed at room temperature by using a magnetic stirrer for 2 h. The mixture was centrifuged at 4100 rpm (i.e. 2400 × *g*) for 20 min and then filtered on 0.22 μm membrane filters (Whatman International Ltd, Maidstone, UK). The sample clean-up was performed using strong cation exchange cartridges, Strata tri-func SCX (100 mg of resin, Phoenemex), preconditioned by sequential treatment with methanol (4 ml × 1 ml) and 1 M phosphoric acid (4 ml × 1 ml). An aliquot of 200 μl of clear sample extract was passed through the preconditioned SCX-SPE cartridge.

After washing with 1 ml of 1 M phosphoric acid, 500 μl of distilled water and 700 μl of 0.04 M borate buffer, pH 9.5, the fraction containing carnosine was eluted with 2 ml of borate buffer and then injected. It was possible to use one cartridge for at least three extractions with identical performances.

Meat samples were prepared by simple suspension of 0.5 g in 10 ml of water. After mixing in an ultrasonic bath and centrifugation at 2400 × *g* for 20 min, the extract was passed on 0.22 μm nylon filters, diluted up 150 times in water, then injected.

3. Results and discussion

3.1. The mobile phase

Sodium hydroxide solutions are usually selected for the separation of sugar and aminoacidic compounds by anion-exchange chromatography. The same type of mechanism was exploited to separate the dipeptide carnosine. Using the hydrophobic and polymeric anion-exchange column AminoPac PA10, a series of NaOH alkaline eluents at concentrations up to 150 mM, also modified by sodium acetate, were investigated. It was found that the separation of carnosine (C) and the related anserine (A) and homocarnosine (H) (see Fig. 1), was very sensitive to the mobile phase composition (Table 1). Acetate ion contributes only towards shortening the retention times without significantly affecting the selectivity. Despite the structural similarity of these carnosine-related peptides, a satisfactory separation was obtained with 100 mM NaOH as eluent (Fig. 1). The difference in the retention times between anserine and the couple homocarnosine and carnosine may be rationalized considering that a weakly acidic –NH– group is contained in the imidazole ring of both homocarnosine and carnosine. Therefore, these last two dipeptides are more strongly retained on the anion-exchange column. The present results were obtained at approximately 25 °C. A more detailed study showed that, as the temperature increases from 15 to 35 °C, retention decreases for all three dipeptides (data not shown). Carnosine exhibits a higher temperature dependence on retention, but the separation between carnosine and homocarnosine does not significantly improve even at 15 °C. All subsequent work was carried out at approximately 25 °C.

Table 1
Retention times (min) of anserine (A), homocarnosine (H) and carnosine (C) as a function of mobile phase composition[a]

Eluent solution	A	H	C
100 mM NaOH + 10 mM NaOAc	4.1	11.8	13.4
150 mM NaOH	4.3	14.3	16.5
100 mM NaOH	4.9	15.2	17.9
80 mM NaOH	5.6	20.1	23.6

[a] Column AminoPac PA10 (250 mm × 2 mm) plus guard (50 mm × 2 mm) at a flow rate of 0.15 ml/min; column temperature, 25 °C; volume injected, 10 μl; potential waveform as listed in Table 2.

Table 2
Potential–time settings of the six-potential step waveform optimized for carnosine detection

Potential vs. Ag|AgCl (V)		Time (s)	Current integration
E_1	−0.20	0.00	
E_1	−0.20	0.04	
E_2	−0.05	0.05	
E_2	−0.05	0.11	Begin
E_3	0.24	0.12	
E_3	0.24	0.41	
E_4	−0.05	0.42	
E_4	−0.05	0.56	End
E_5	−2.00	0.57	
E_5	−2.00	0.58	
E_6	0.60	0.59	
E_1	−0.20	0.60	

3.2. Electrochemical detection in pulsed amperometry

Not being the conventional three-potential waveform very suitable to determine low concentrations of amino compounds [29], a series of integrated potential waveforms, generally applied to detect amino acids and similar compounds at a gold working electrode in alkaline solutions [30–32] were investigated. As the six-potential waveform proposed by Clarke et al. [30] offers the highest sensitivity (not shown), it was chosen and successively optimized for the amperometric detection of carnosine by varying the detection potential (E_3) in the potential waveform from +0.18 to +0.34 V versus Ag|AgCl, in 20 mV steps. The best results in terms of sensitivity and signal-to-noise ratio were obtained with a potential of +0.24 V and this value was applied in all subsequent chromatographic runs. The optimized waveform parameters are detailed in Table 2.

3.3. Solid-phase isolation and recovery

In an attempt to decrease the number of interfering peaks from feed samples, a cation-exchange cleanup was investigated. Isolation efficacy and recovery of carnosine was verified by solid phase extraction, using 200 μl of a 60 μM carnosine water solution passed through a SCX cartridge, previously activated by methanol (4 ml × 1 ml) and 1 M phosphoric acid (4 ml × 1 ml). Upon washing with water, 2 ml of borate buffer eluate was injected and analyzed by HPAEC–IPAD. Recoveries of carnosine were evaluated by comparison of peak area to that obtained from an equivalent carnosine solution, not passed through the cartridge. No significant differences were observed, confirming a carnosine recovery higher than 94.6% ($n = 5$). Moreover, an extraction efficiency of approximately 96 ± 2% ($n = 5$) was also determined from the recovery of carnosine in spiked compared to unspiked feed samples, treated in the same way throughout the whole procedure. Mean intraday assay

precision ($n = 5$), expressed as coefficient of variation, was 3.4%. Interassay precision ($n = 7$) was 4.2%.

3.4. Repeatability of retention and calibration data

When using a 10 μM carnosine solution, the relative standard deviations (R.S.D.s) of retention times were lower than 1.8% ($n = 7$) for the same day and increased up to 3.4% for experiments repeated on five different days. The R.S.D.s of peak areas were better than 2.7%. The correlation between peak area and carnosine concentration was examined in the range 0.1–100 μM ($r = 0.9998$). The standard deviation (S.D.) of slope and intercept was estimated at the 95% confidence level. The LOD, determined at a signal-to-noise ratio of 3, was 0.1 μM, which corresponded with an injection of 0.23 ng of carnosine.

3.5. Assay of carnosine in feed samples

Five feed samples, three for cattle and two for sheep, were analyzed. As already mentioned, good separation of carnosine from endogenous compounds present in such samples was found with 100 mM NaOH as a mobile phase. Fig. 2 shows results for two samples of the same bovine feed both without and with spiking (1 mg of carnosine for 2.5 g of sample) prior to sample clean-up. The chromatogram of the feed sample is interference-free in the time-window where carnosine elutes. No carnosine peak

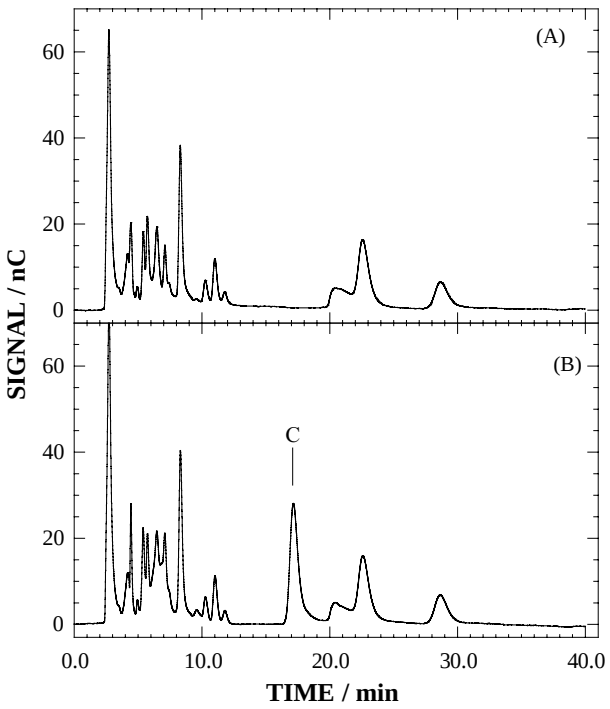

Fig. 2. HPAEC–IPAD separations of (A) bovine feed sample and (B) sample spiked with an amount of 0.04% of carnosine prior to sample clean-up. No carnosine was found in the animal feed examined (panel A). Other experimental conditions as in Fig. 1.

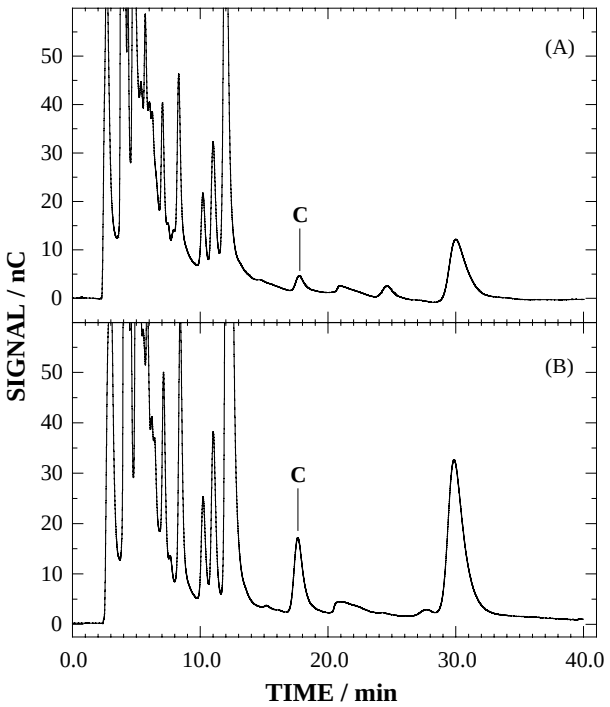

Fig. 3. HPAEC–IPAD of two commercial feeds: (A) for adult dogs and (B) for one-year cats. Other conditions as in Fig. 1.

was observed in the other analyzed bovine and ovine feeds, which confirms the absence of processed animal proteins in the investigated samples. Taking into account the sensitivity of the HPAEC–IPAD method and the high level of recovery throughout the SPE protocol, the proposed assay allows carnosine to be determined down to 5 μg/g of feed. The routine analytical determination of carnosine in feed samples is, thus, possible. It should be mentioned, however, that the separation of anserine is subject to interferences from early eluting compounds and consequently its quantification is not possible under the present experimental conditions.

Fig. 3 compares the chromatograms of two commercial feeds, for adult dogs and cats. In both chromatograms, the presence of a peak whose retention time matches that of carnosine was observed, suggesting its presence in the meat-based animal feeds. Other injections were made with samples that were spiked with small amounts of carnosine for peak identification. Since interference from other compounds in feed samples may be a problem, the use of a second type of anion-exchange column, the CarboPac PA1 (microbore format), was evaluated. The same outcome was confirmed under the following experimental conditions, 100 mM NaOH eluent at a flow rate of 0.25 ml/min. The mean content of carnosine was evaluated by standard additions and was found to be 74 ± 5 and 148 ± 15 μg/g of feed for dogs and cats, respectively. The peak area RSD of five corresponding injections of these samples was 5 and 4%, feed for dogs and cats, respectively. These results are extremely encouraging and strongly suggest that the combination of HPAEC with pulsed amperometry is a

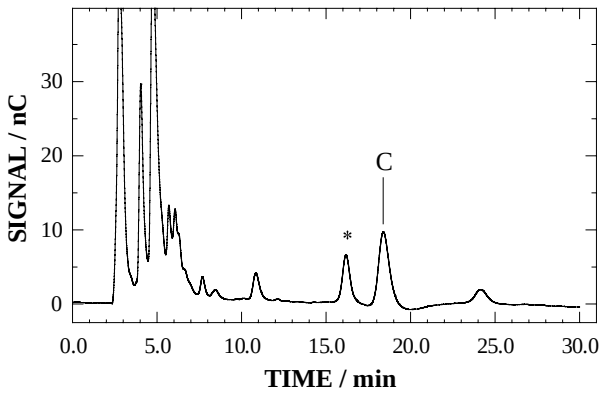

Fig. 4. HPAEC–IPAD separation of a lyophilized lamb meat sample, diluted 150-fold before injection. The peak marked with asterisk corresponds to taurine, identified by standard addition. Other conditions are those described in Fig. 1.

very practical assay for carnosine quantitation in animal feeds.

The procedure was also applied to the analysis of carnosine in meat samples. The good separation is illustrated in Fig. 4, which shows a representative chromatogram of a sample of lyophilized lamb meat. Anserine, even if present, cannot be quantified being not well separated from the other peaks near the solvent front. Homocarnosine is presumably absent, while the mean content of carnosine was evaluated as the average of three measurements performed on different amounts of the same sample. Analogous considerations were applied to a lyophilized beef sample (not shown). The carnosine content was higher than for the lamb sample, 310 ± 30 and 140 ± 20 mg for 100 g of product, respectively. These findings agree with those of other authors who have observed that amount of carnosine within not-lyophilized meats is 50–300 mg/100 g of muscular tissue [1].

4. Conclusions

As carnosine determination would represent a useful alternative to the official microscopic examination of feed analysis, the developed HPAEC–IPAD method seems to be very suitable to assess the presence of processed animal proteins in animal feeds. The method is convenient, sensitive, and reliable and can be considered a significant improvement over the established chromatographic techniques in which pre-column derivatization is employed. This method is also a valid and effective methodology for carnosine determination in meat and could be useful to increase the knowledge on the biological roles of carnosine in vertebrate systems.

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References

[1] E.A. Decker, Nutr. Rev. 53 (1995) 49.
[2] M. Dunnett, R.C. Harris, M.Z. Soliman, A.S.A. Suwar, Res. Vet. Sci. 62 (1997) 213.
[3] W. Gulewitsch, S. Amiradzibi, Ber. Disch. Chem. Ges. 33 (1990) 1902.
[4] F. Margolis, Science 184 (1974) 909.
[5] A.A. Boldyrev, A.M. Dupin, A.Y. Bunin, M.A. Babizhayev, S.E. Severin, Biochem. Int. 15 (1987) 1105.
[6] J.L. Jay, D.J. Miller, J.D. Morrison, J.J. O'Dowd, Meeting Abstr. Physiol. Lond. 420 (1990) 155.
[7] E.C. Bate-Smith, J. Physiol. 92 (1938) 336.
[8] R.C. Harris, D.J. Marlin, M. Dunnett, D.H. Snow, E. Hultman, Comp. Biochem. Physiol. 97A (1990) 249.
[9] S.H. Snyder, Science 209 (1980) 976.
[10] S. Zhou, E.A. Decker, J. Agric. Food Chem. 47 (1999) 51.
[11] M.A. Babizhayev, M.C. Seguin, J. Gueyne, R.P. Evstigneeva, E.A. Ageyeva, G.A. Zheltukhina, Biochem. J. 304 (1994) 509.
[12] T.A. Dahl, W.R. Midden, P.E. Hartman, Photochem. Photobiol. 47 (1988) 357.
[13] M.A. Babizhayev, Biochim. Biophys. Acta 1004 (1989) 363.
[14] EC Council Decision 766/2000, Off. J. Eur. Communities L306 (2000) 32.
[15] Commission Directive 98/88/EG, Off. J. Eur. Communities L318 (1998) 45.
[16] J. Schönherr, J. Agric. Food Chem. 50 (2002) 1945.
[17] M. Kuroda, R. Ohtake, E. Suzuki, T. Harada, J. Agric. Food Chem. 48 (2000) 6317.
[18] J. Wideman, L. Brink, S. Stein, Anal. Chem. 86 (1998) 670.
[19] E. Kasziba, J. Chromatogr. 432 (1998) 315.
[20] K. Teahon, J.M. Rideout, Biomed. Chromatogr. 6 (1992) 16.
[21] P.R. Carnegie, M.Z. Ilic, M.O. Etheridge, M.G. Collins, J. Chromatogr. 261 (1983) 153.
[22] J.E. Plowman, E.A. Close, J. Sci. Food Chem. 45 (1988) 69.
[23] R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Anal. Chem. 70 (1998) 1496.
[24] W.R. LaCourse, D.C. Johnson, Anal. Chem. 65 (1993) 50.
[25] P. Jandik, A.P. Clarke, N. Avdalovic, D.C. Andersen, J. Cacia, J. Chromatogr. B 732 (1999) 193.
[26] C. Thiele, M.G. Gänzle, R.F. Vogel, Anal. Biochem. 310 (2002) 171.
[27] T.R.I. Cataldi, C. Campa, G.E. De Benedetto, Fresenius J. Anal. Chem. 368 (2000) 739.
[28] T.R.I. Cataldi, D. Nardiello, J. Agric. Food Chem. 51 (2003) 3737.
[29] W.R. LaCourse, Pulsed Electrochemical Detection in High-Performance Liquid Chromatography, Wiley, New York, 1997.
[30] A.P. Clarke, P. Jandik, R.D. Rocklin, Y. Liu, N. Avdalovic, Anal. Chem. 71 (1999) 2774.
[31] S. Cavalli, N. Cardellicchio, J. Chromatogr. A 706 (1995) 429.
[32] L.E. Welch, W.R. LaCourse, D.A. Mead Jr., D.C. Johnson, T. Hu, Anal. Chem. 61 (1989) 555.