Improved methods of analysis for betaines in Ascophyllum nodosum and its commercial seaweed extracts

MacKinnon, Shawna L.; Hiltz, David; Ugarte, Raul; Craft, Cheryl A.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s10811-009-9483-0
Journal of Applied Phycology, 22, 4, pp. 489-494, 2010-01-01

J Appl Phycol (2010) 22:489–494
DOI 10.1007/s10811-009-9483-0

Improved methods of analysis for betaines in *Ascophyllum nodosum* and its commercial seaweed extracts

Shawna L. MacKinnon · David Hiltz · Raul Ugarte · Cheryl A. Craft

Received: 23 June 2009 / Revised and accepted: 30 October 2009 / Published online: 27 November 2009

Abstract Beneficial effects of seaweeds and their extracts on crop performance have been attributed to a variety of compounds, including the betaines which are quaternary ammonium betaines. Methods of analysis of betaines published thus far suffer from low sensitivity, lack of baseline separation of individual betaines and from interference from other sample constituents. A rapid cleanup protocol and a sensitive LC-MS/MS method of analysis were developed to afford baseline separation of four betaines in the brown alga *Ascophyllum nodosum* and its commercial seaweed extract. Using this method, the presence of glycine betaine, δ-aminovaleric acid betaine, γ-aminobutyric acid betaine and laminine in *A. nodosum*, and commercial extracts derived from *A. nodosum*, were confirmed and quantified. The major betaine present was γ-aminobutyric acid betaine accounting for 0.008–0.014% of the dry weight of the seaweed and 0.014–0.027% of the dry weight of the commercial extracts. Seasonal variation in betaine content was observed. Differences in the total betaine content were observed between *A. nodosum* of the yellow (0.011–0.017% dry weight) and the olive green (0.017–0.021% dry weight) coloured morphologies.

Keywords Glycine betaine · δ-aminovaleric acid betaine · γ-aminobutyric acid betaine · Laminine · LC-MS/MS analysis

S. L. MacKinnon (✉) · C. A. Craft
Institute for Marine Biosciences,
National Research Council of Canada,
1411 Oxford Street,
Halifax, NS, Canada B3H 3Z1
e-mail: shawna.mackinnon@nrc-cnrc.gc.ca

D. Hiltz · R. Ugarte
Acadian Seaplants Limited,
30 Brown Ave.,
Dartmouth, NS, Canada B3B 1X8

Abbreviations

GB	Glycine betaine
AVAB	δ-Aminovaleric acid betaine
ABAB	γ-Aminobutyric acid betaine
LAM	Laminine

Introduction

Historically, brown seaweeds such as *Ascophyllum nodosum* have been applied directly to the soil, either freshly harvested or as dried ground seaweed meal, primarily as a soil conditioner or source of organic matter (Temple and Bomke 1988). More recently, aqueous and alkaline extracts prepared from a variety of commercially available algae have been used in agriculture and horticulture systems as foliar sprays, soil drenches or often a combination of both. The beneficial effects of seaweed extract application on crop performance have been attributed to a variety of constituents, including betaines (Blunden et al. 1981). These naturally occurring quaternary ammonium compounds can act as osmolytes and/or affect gene expression, therefore improving plant tolerance to stresses such as temperature extremes, drought and salinity (Mason and Blunden 1989; Hayashi et al. 1997; Holmström et al. 2000; Sakamoto et al. 2000; Park et al. 2004; Yang et al. 2007).

Over 13 betaine analogs have been identified in Chlorophycota (green), Chromophycota (brown) and Rhodophycota (red) seaweeds using thin-layer chromatography and NMR techniques (Blunden et al. 1992). The isolated yield of each betaine ranged from 0% to 2% per algal dry weight, with the lowest betaine levels being reported in the Phaeophyceae. Seaweed biomass and commercial extracts prepared from *A. nodosum* and *Fucus* and *Laminaria* species have been found to contain glycine betaine, γ-aminobutyric acid betaine, δ-aminovaleric acid betaine

and laminine (Fig. 1; Tyihák et al. 1994; Blunden et al. 1984).

Early analytical methods aimed at the quantification of betaines in plants, seaweeds or commercial seaweed extracts included the use of thin-layer chromatography (Müller and Eckert 1989), thin-layer electrophoresis (Gorham et al. 1981) and non-specific precipitation reagents (Barak and Tuma 1979; Bao et al. 1989). The development of a high-performance liquid chromatography (HPLC)-based approach to betaine analysis has been ongoing since the late 1970s (Dupuy 1978). Betaines do not fluoresce appreciably and can only be detected at low UV wavelengths (190–220 nm) as they lack a strong chromophoric moiety (Zamarreño et al. 1997). Attempts at increasing the detection limits of betaines using derivatization reagents have given unsatisfactory results because of the lack of reactivity of the betaines towards some reagents (Lever et al. 1992). Proton magnetic resonance (^{1}H NMR) has also been developed as a method of betaine analysis for seaweed extracts and plant material (Blunden et al. 1986) but has the limitation that it cannot be used to quantify the levels of betaines that are present as minor components (Tyihák et al. 1994).

The LC-MS/MS method described herein quantifies betaines in brown seaweeds and commercial seaweed extract products directed at the agricultural market. The rapid sample preparation and cleanup protocols coupled with the sensitivity and selectivity of LC-MS/MS make this method suitable for the routine analysis of glycine betaine (GB), γ-aminobutyric acid betaine (ABAB) and δ-aminovaleric acid betaine (AVAB) and laminine (LAM) in both *A. nodosum* and its commercial seaweed extracts.

Materials and methods

Glycine betaine (catalogue no. 14290; Fluka Chemicka/Biochemika, Switzerland), LAM (Nε,Nε,Nε-trimethyl lysine; catalogue no. T 1660; Sigma-Aldrich Canada Ltd., Canada), ABAB (synthesised) and AVAB (synthesised) were subjected to quantitative NMR analysis before use. Deuterated GB (d_{11}-glycine betaine) was purchased from Cambridge Isotope Laboratories Inc. (catalogue no. DLM 407-1, Andover, USA). *Ascophyllum nodosum* seaweed used in this study was harvested at Shag Harbour (site 1) and Lobster Bay (site 2) in southwestern Nova Scotia, Canada. Seaweed samples were collected at low tide during May, August and December 2005, stored in a cooler containing ice and immediately transported to the laboratory. Five batches of commercial extracts of *A. nodosum* that were manufactured using *A. nodosum* that had been harvested from sites 1 and 2 were provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. Four of the batches had been manufactured in 2005 from May to December, whilst one had been manufactured in 2001.

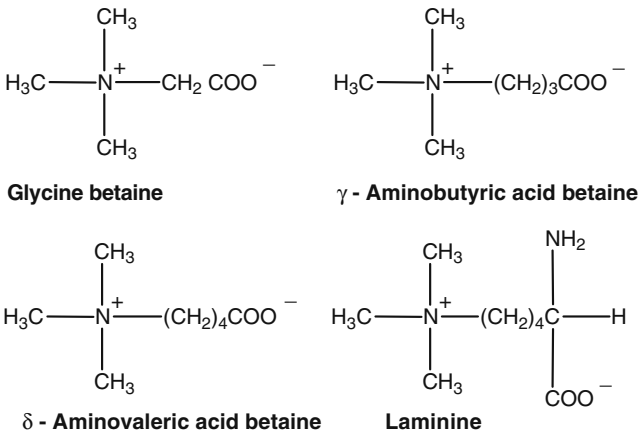

Fig. 1 Betaine structures

Preparation of γ-aminobutyric acid betaine and δ-aminovaleric acid betaine The ABAB and AVAB standards used in this study were synthesised and semi-purified by crystallisation from methanol/ether using a method previously described in the literature (Benoiton and Chen 1976). Further purification was accomplished using a three-ion exchanger column system that utilised a strong anion, a weak cation and strong cation ion exchange columns arranged in series. The procedure used to purify the betaines differed only from that reported by Bessieres et al. (1999) in that Permutit Q (50-100 mesh, H+ form) was used in place of the AG50W resin (strong cation exchange resin). The eluted betaines were evaporated to dryness using a rotary evaporator and then freeze-dried. Quantitative NMR was used to determine the purity of the betaine standards.

Seaweed sample preparation Fresh *A. nodosum* samples, which were previously cleaned of other algal species and marine organisms, were coarsely ground with liquid nitrogen using a mortar and pestle and then ground into a fine powder using dry ice and an IKA M20 grinder (IKA Works Inc. USA). Samples were stored at −80°C prior to analysis.

The ground seaweed (0.250 g) or soluble seaweed extract (0.100 g) samples were heated with 2.00 mL of methanol for 2 h at 78°C in a 5-mL Reacti-vial (catalogue no. PI-13223; ThermoFisher, Canada). The methanol extract was removed, the sample extracted once more as above and then extracted two times with 2.00 mL of methanol for 1 h at 78°C. The resulting methanol extracts were combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 5.00 mL of 0.2% formic acid containing 5 mM ammonium formate and loaded onto a C_{18} SPE column (3 cc (200 mg), C_{18}

cartridge, catalogue no. WAT054945; Waters, Canada) which had been preconditioned by washing with 5 mL of methanol followed by 15 mL of 0.2% formic acid/5 mM ammonium formate. The betaine-containing fraction was obtained by eluting the column with 15 mL of 0.2% formic acid/5 mM ammonium formate. This fraction was evaporated to dryness under a stream of nitrogen and then redissolved in 2.00 mL of methanol in preparation for LC-MS/MS analysis.

Experiments that were directed at developing the optimum extraction conditions and recoveries of betaines were conducted in triplicate with triplicate injections onto the LC-MS/MS. The optimum extraction of GB, ABAB, AVAB and LAM from *A. nodosum* and *A. nodosum* extract samples was accomplished by monitoring the quantities of betaines extracted using each solvent system. Recovery experiments directed at evaluating the use of OASIS MCX, AG50W-8X and C_{18} SPE supports to further purify a betaine-containing fraction were conducted using betaine standards. The recovery of glycine betaine from the seaweed matrix was evaluated by adding d_{11} glycine betaine to the seaweed sample that was then subjected to the developed sample preparation procedure. The concentration of d_{11} glycine betaine added to the sample was similar to the level of glycine betaine present in the starting sample.

The dry matter content of wet seaweed samples was determined by freeze drying a known weight of seaweed followed by placing the samples in a convection oven at 95°C for 16 h to obtain the dry weight of the sample. Commercial seaweed extract samples were dried in a convection oven at 95°C for 16 h to determine their moisture content.

LC-MS/MS analysis LC-MS/MS analysis was performed on a *Sciex* API 4000 Triple Quadrapole Mass Spectrometer (SCIEX, Canada) coupled to an Agilent 1100 HPLC (Palo Alto, USA). The instrument was equipped with Turbo IonSpray source and operated in a positive multiple reaction monitoring mode using the following precursor-to-product ion transitions: 118.2>58.2 *m/z* for GB, 159.9>101.1 *m/z* for AVAB, 146.1>87.0 *m/z* for ABAB and 188.9>83.9 *m/z* for LAM. The MS was operated with the electrospray voltage set to 5,000 V, the collision energy at 30 V and the nebulizer gas temperature at 300°C. Nitrogen was used for both the nebulizer and curtain gas. Chromatographic separation of the betaines was achieved using an Alltima HP HILIC Expedite™ MS column (4.6×20 mm; 1.5 μm (particle size); catalogue no. 86472; Grace, USA) operated at 40°C. The column was eluted isocratically at a flow rate of 1.0 mL/min from 0 to 7 min with 15% A:85% B and then from 7 to 15 min with 35% A:65% B. Aqueous solvent A contained 0.01% formic acid and 5 mM ammonium formate, whereas solvent B contained 95% acetonitrile/5% water (0.01% formic acid with 5 mM ammonium formate). Under these conditions, GB, AVAB, ABAB and LAM eluted at 1.6, 5.2, 6.3 and 9.3 min, respectively.

Results

LC-MS/MS analysis of betaine content Investigations into the limits of detection using standards were preliminary but revealed that GB, ABAB, AVAB and LAM could be detected at nanogram levels using this LC-MS/MS method, thus permitting the detection and confirmation of the presence of specific betaines on the basis of their molecular ion and fragment ion peaks. Hydrophilic interaction chromatography (HILIC) afforded baseline separation of a mixture of the betaine standards GB, ABAB, AVAB and LAM in a run time of 15 min. The same baseline separation was achieved with either fresh *A. nodosum* and an *A. nodosum* commercial extract (Figs. 2 and 3), showing that the developed LC-MS/MS method can be used for both products.

Optimization of the betaine extraction conditions for *A. nodosum* and the commercial *A. nodosum* extract samples was explored using methanol, 20% H_2O/acetone, 20% H_2O/methanol and 20% H_2O/acetonitrile as solvents at different temperatures. Our investigations showed that five methanol extractions at 78°C gave the highest recovery of the four betaines of interest. Partial purification of the methanol extracts with OASIS MCX and AG50W-8X resins gave good recovery of GB, AVAB and ABAB in our experiments but very poor recovery of LAM (2–5%). Laminine was found to be stable at 78°C for 16 h in methanol but degraded when dissolved in 3 M ammonium hydroxide at room temperature. Elution of the extract-loaded C_{18} SPE with 0.1% triethylamine yielded a 18% recovery of LAM; however, elution with 0.2 % formic acid resulted in a >87 % recovery. Results from the addition of

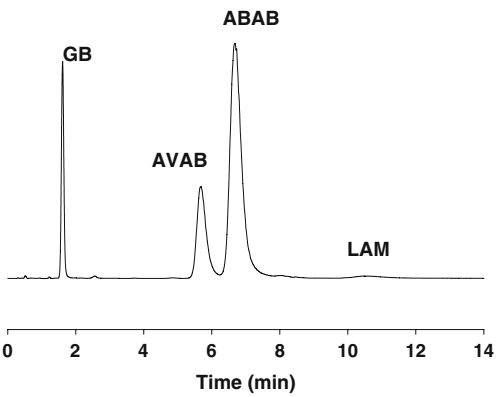

Fig. 2 LC-MS/MS profile of betaines in *A. nodosum*

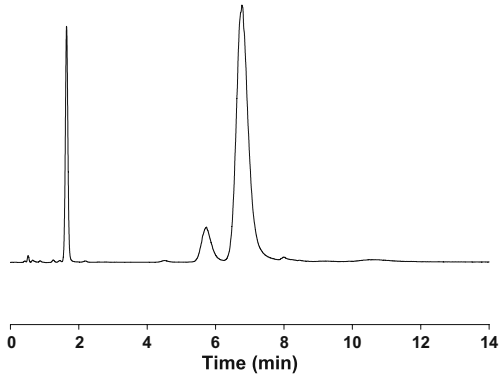

Fig. 3 LC-MS/MS profile of betaines in a commercial extract derived from *A. nodosum*

d-$_{11}$GB to a sample prior to extraction showed that betaine recovery exceeded 95%. Semi-purification of the combined methanol extracts for LC-MS/MS analysis was therefore accomplished using C_{18}SPE columns and not using ion exchange columns as used by other researchers (Blunden et al. 1992; Tyihák et al. 1994).

Betaine content of A. nodosum samples The developed method was used to survey the betaine content of *A. nodosum* at two collection sites and at three collection times in southern Nova Scotia, Canada (Table 1). In all the collections, ABAB was the major betaine present in the seaweed samples, accounting for 0.008–0.014% of the dry weight of the seaweed. Aminovaleric acid betaine was present at levels three to five times less than ABAB but greater than either GB or LAM. Laminine was present at 2.78–8.90 μg g^{-1} dry weight of seaweed (0.0003–0.001% dry weight). Higher levels of LAM and GB were observed overall in site 2 *A. nodosum* samples. Seasonal variation in betaine content was observed in the GB content in site 2 but not in site 1 data and in the ABAB content in site 1 but not in site 2 data. The total betaine content at site 1 changed seasonally, but that of site 2 change very little with season. The total betaine content at site 2 was considerably higher than that at site 1 for May and August, which coincides with the time in which the seaweed is undergoing active growth.

Betaine content of commercial extracts of A. nodosum Analysis of the betaine content of commercial seaweed extracts prepared from *A. nodosum* was also undertaken (Table 2). The major betaine present in all five samples was ABAB at a level of 136–266 μg g^{-1} dry weight (0.014–0.027% dry weight). The AVAB content of the commercial extracts was only slightly lower in content, 117–203 μg g^{-1}dry weight (0.012–0.020% dry weight), than ABAB, which is different than that observed in *A. nodosum* (Table 1). The level of GB varied from 34.5 to 61.6 μg g^{-1} dry weight (0.003–0.006% dry weight) and was present in higher amounts than LAM.

Discussion

LC-MS/MS analysis of betaine content The analysis of betaines using traditional GC and LC methods often requires derivatization as the compounds of interests are not volatile, do not fluoresce and only exhibit a weak absorption at 190–220 nm. These derivatization steps can

Table 1 Seasonal betaine content in two morphological types of *A. nodosum* on a dry weight basis (±standard deviation, n=9)

	Glycine betaine (μg g^{-1}) (%)	γ-Aminobutyric acid betaine (μg g^{-1}) (%)	δ-Aminovaleric acid betaine (μg g^{-1}) (%)	Laminine (μg g^{-1}) (%)	Total betaine (μg g^{-1}) (%)
Site 1					
May	8.66±1.73	110±11	26.2±2.1	3.43±0.75	148±11
	(0.001)	(0.011)	(0.003)	(0.0003)	(0.015)
August	7.41±0.65	74.8±2.4	23.1±0.7	2.78±0.32	108±3
	(0.001)	(0.008)	(0.002)	(0.0003)	(0.011)
December	6.97±0.68	130±6	27.1±1.5	5.96±0.80	170±6
	(0.001)	(0.013)	(0.003)	(0.001)	(0.017)
Site 2					
May	21.9±3.8	140±8	34.3±1.3	8.90±1.25	205±9
	(0.002)	(0.014)	(0.003)	(0.001)	(0.021)
August	5.63±0.44	121±7	47.3±2.7	6.76±1.49	181±8
	(0.001)	(0.012)	(0.005)	(0.001)	(0.018)
December	23.4±2.5	120±8	24.7±2.0	5.67±0.87	174±9
	(0.002)	(0.012)	(0.002)	(0.001)	(0.017)

Table 2 Variation in betaine content of five *A. nodosum*-derived commercial extracts on a dry weight basis (±standard deviation, $n=9$)

	Glycine betaine ($\mu g\ g^{-1}$) (%)	γ-Aminobutyric acid betaine ($\mu g\ g^{-1}$) (%)	δ-Aminovaleric acid betaine ($\mu g\ g^{-1}$) (%)	Laminine ($\mu g\ g^{-1}$) (%)
1	34.5±1.5	244±7	157±6	4.38±1.93
	(0.003)	(0.020)	(0.016)	(0.0004)
2	45.8±1.3	136±4	145±4	10.0±0.9
	(0.005)	(0.014)	(0.015)	(0.001)
3	61.6±3.1	211±3	152±2	4.34±0.43
	(0.006)	(0.021)	(0.015)	(0.0004)
4	54.3±1.4	186±6	117±3	4.56±0.21
	(0.005)	(0.019)	(0.012)	(0.0005)
5	36.2±1.6	266±6	203±6	27.1±2.0
	(0.004)	(0.027)	(0.020)	(0.003)

be time-consuming and may lead to poor recoveries. Other methods of detection, such as quantitative proton NMR and optimum-performance laminar chromatography, require significant purification prior to analysis in order to eliminate potential interferences (Tyihák et al. 1994). We developed a sample extraction and cleanup protocol for an improved method for the detection and quantification of GB, ABAB, AVAB and LAM in fresh seaweed and dried seaweed extracts using HILIC column technology and LC-MS/MS analysis.

Betaine content of A. nodosum Recently, the betaine content of *A. nodosum* was surveyed using NMR analysis (Blunden et al. 2009). The dominant betaine in the study was found to be ABAB which was present at levels of 0.02–0.07% dry weight, which is higher than our present observations. The sizeable concentration difference between AVAB and ABAB seen in our study was not observed in the study utilising the NMR analysis technique (Blunden et al. 2009) which showed AVAB and ABAB to be present at similar levels. An explanation for the differing results between the two studies is unclear and needs to be further investigated.

Laminine was first detected in *A. nodosum* using thin-layer chromatography (Blunden et al. 1985), but the level of LAM has not been reported since using NMR or other forms of analysis. This may be due to the instability of LAM in the elution solvents used in the "cleanup" protocols to prepare the samples for analysis. As a result, LAM may have been present at levels below the detection limits of the earlier analytical method. The presence of GB in *A. nodosum* has not been previously reported. Because *A. nodosum* samples were cleaned of other seaweeds, including *Fucus* species, prior to analysis, the authors are confident in reporting the presence of GB in *A. nodosum*.

The total betaine content of *A. nodosum* collected at site 2 (0.017–0.021% dry weight) was found to be higher than that of site 1 (0.011–0.017% dry weight), which was not surprising as the seaweed collected at the two sites were different morphologically (Fig. 4). Seaweed collected at site 1 was yellow whilst that at the second site was olive green throughout the year. Previous observations also showed that the *A. nodosum* at site 1 had a growth rate of 3–5 cm year whilst that of site 2 had a growth rate of 13–17 cm year. Overall, no clear trend was seen in the levels of individual betaines and total betaine content of the *A. nodosum* for each site that indicated that the betaine content varied with time of harvest.

Betaine content of commercial extracts of A. nodosum All four betaines have been reported previously in commercial liquid seaweed extracts prepared from *A. nodosum* and analysed using overpressured liquid chromatography (Tyihák et al. 1994). As shown in the Tyihák et al. reference, ABAB was present at levels double that of AVAB, and GB was at roughly the same level as AVAB. The difference between these results and the present LC-MS/MS study could be due to variations in processing methods and differences in the betaine content and profile of the *A. nodosum* biomass used to prepare the commercial extracts. Further comparisons between the two studies were difficult because the dry weight of the liquid seaweed extract was not available.

Fig. 4 *Ascophyllum nodosum* collected at Shag Harbour (site 1—*right* (20 cm in length)) and Lobster Bay (site 2—*left*) in southwestern Nova Scotia, Canada

The rapid sample preparation and cleanup protocols coupled with the sensitivity and selectivity of LC-MS/MS make the presented method suitable for the routine analysis of betaines in both *A. nodosum* seaweed and commercial products prepared from *A. nodosum*. Studies of LAM in different solvents highlighted its instability and the need to monitor its recovery whilst developing a quantitative method of analysis. The results from our study suggest that deuterated LAM should be used as an internal standard to validate LAM quantitation. Such a standard is not commercially available, but the authors are presently pursuing its synthesis. While this study focused on the analysis of betaines in *A. nodosum*, it is expected that the LC-MS/MS method will be applicable to the analysis of betaines in other seaweed species.

Acknowledgements This work was supported by The Institute for Marine Biosciences and a grant to Acadian Seaplants Limited from the National Research Council of Canada–Industrial Research Assistance Program (NRC-IRAP).

References

Bao W, Gao S, Fan Z, Jiang Z (1989) Isolation and identification of betaine from beet molasses and its quantitative determination. J Shenyang Coll Pharm 6:12–15

Barak AJ, Tuma DJ (1979) Simplified procedure for determination of betaine in liver. Lipids 14:860–863

Benoiton NL, Chen FMF (1976) The synthesis of amino-acid derivatives and peptides containing *N*,*N*-dimethylamino and *N*,*N*,*N*-trimethylamino groups. Proceedings of the 14th European Peptide Symposium, pp 149–152

Bessieres M-A, Gibon Y, Lefeuvre JC, Larher F (1999) A single-step purification for glycine betaine determination in plant extracts by isocratic HPLC. J Agric Food Chem 47:3718–3722

Blunden G, El Barouni MM, Gordon SM, McLean WFH, Rogers DJ (1981) Extractions, purification and characterization of Dragendorff-positive compounds from some British marine algae. Bot Mar 24:451–456

Blunden G, Rogers DJ, Barwell CJ (1984) Biologically-active compounds from British marine algae. In: Krogsgaard-Larsen P, Brogger Christensen S, Kofod H (eds) Natural products and drug development, Alfred Benzon Symposium 20. Munksgaard, Copenhagen, pp 179–190

Blunden G, Gordon SM, Smith BE, Fletcher RL (1985) Quaternary ammonium compounds in species of the Fucaceae (Phaeophyceae) from Britain. Br Phycol J 20:105–108

Blunden G, Cripps AL, Gordon SM, Mason TG, Turner CH (1986) The characterization and quantitative estimation of betaines in commercial seaweed extracts. Bot Mar 29:155–160

Blunden G, Smith BE, Irons MW, Yang M-H, Roch OG, Patel AV (1992) Betaines and tertiary sulphonium compounds from 62 species of marine algae. Biochem Syst Ecol 20(4):373–388

Blunden G, Currie M, Mathe J, Hohmann J, Critchley A (2009) Variations in betaine yields from marine algal species commonly used in the preparation of seaweed extracts used in agriculture (abstract). Phycologist 76:14

Dupuy P (1978) Analytical recognition of chaptalization (addition of sugar to wine after fermentation). Ann Nutr Aliment 32:1123–1132

Gorham J, Coughlan SJ, Storey R, Jones RGW (1981) Estimation of quaternary ammonium and tertiary sulphonium compounds by thin-layer electrophoresis and scanning reflectance densitometry. J Chromatogr 210:550–554

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase: accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Holmström KO, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51:177–185

Lever M, Bason L, Leaver C, Hayman CM, Chambers ST (1992) Same-day measurement of glycinebetaine, carnitine and other betaines in biological material. Anal Biochem 205:14–21

Mason TG, Blunden G (1989) Quaternary ammonium and tertiary sulphonium compounds of algal origin as alleviators of osmotic stress. Bot Mar 32:313–316

Müller H, Eckert H (1989) Simultaneous determination of monoethanolamine and glycine betaine in plants. J Chromatogr 479:452–458

Park E-J, Jeknić Z, Sakamoto A, DeNoma J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. Plant J 40:474–487

Sakamoto A, Valverde R, Alia, Chen THH, Murata N (2000) Transformation of *Arabidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. Plant J 22:449–453

Temple WD, Bomke AA (1988) Effects of kelp (*Macrocystis integrifolia*) on soil chemical properties and crop response. Plant Soil 105:213–222

Tyihák E, Blunden G, Ma Y-C (1994) Quantitative estimation of betaines in commercial seaweed extracts using overpressured layer chromatography. J Appl Phycol 6:469–473

Yang X, Wen X, Gong H, Lu Q, Yang Z, Tang Y, Liang Z, Lu C (2007) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225:719–733

Zamarreño A, Cantera RG, Garcia-Mina JM (1997) Extraction and determination of glycine betaine in liquid fertilizers. J Agric Food Chem 45:774–776