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A Review of Extraction and Analysis: Methods for Studying Osmoregulants in Plants

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Abstract

Compatible osmolytes are substances produced by plants exposed to stressful environmental conditions. These protect plants during stress by performing several functions including scavenging of free radicals and maintenance of osmotic balance. The three most commonly examined include proline, mannitol and glycine betaine. Extraction and analysis of osmolytes are essential steps for a number of applications including estimation of the potential of new breeds, genetically engineered plants, and plants exposed to extreme environmental conditions. A wide range of extracting solvents have been used for the three key osmoregulants, and rarely is the selection of the extracting solvent experimentally determined. Furthermore, in many studies involving two or more osmolytes each is extracted individually. Similarly, there are a variety of methods reported for the quantification of these compounds. As with extraction, a separate method is often applied in quantifying each osmoregulant. Therefore, the aim of this review is to provide a detailed overview of the available methods for extraction and quantification of compatible osmolytes for the study of plant stress. Furthermore, the methods available for simultaneous extraction and quantification of key osmoregulants have been described.

Keywords: Compatible osmolytes; Proline; Betaine; Mannitol; Extraction; Analysis; HPLC; GC; Capillary electrophoresis

Introduction

Plants rely on gradients in water potential from the external environment (less negative) to the internal components (more negative) of the plant/cell in order to continue to absorb water and maintain cell turgor and growth. When this gradient is interrupted, and the water potential outside the plant becomes more negative, the plant becomes stressed. This can occur on a diurnal basis and results in plants responding by reducing water use via short term mechanisms such as closing stomata and hence reducing photosynthesis. However, when the stress is prolonged, due to adverse conditions such drought, high soil salinity or water access restriction through low temperatures (i.e., water is frozen), plants can accumulate excess ions, particularly in cell vacuoles, to reduce cellular osmotic potential. The continued accumulation of such ions will eventually lead to toxicity problems (especially when the ions are normally toxic e.g., excess Na^+ or Cl^-) and excess ions start to accumulate in the cell's cytoplasm. To counteract this toxicity, plants can produce excess compatible osmolytes (COs) which have properties that can act in a manner similar to inorganic ions (i.e., lower the osmotic potential of cells) [1,2] or function in other ways that help to protect cellular integrity. Most importantly, they are able to prevent protein denaturation that can occur with ionic imbalances [3].

Compatible osmolytes (COs) are low molecular weight metabolites and include sugars (e.g., sucrose and trehalose), sugar alcohols (e.g., mannitol), amino acids (e.g., proline and glutamate), quaternary ammonium compounds (e.g., glycine betaine and carnitine) and tetrahydropyrimidines (e.g., ecotine and hydroxyecotine) [4]. Their functions are variable but include, along with those above, stabilization of sub-cellular structures [1], regulation of co-enzymes [2] and scavenging of free radicals to prevent membrane degradation [2,5]. The significance of each CO varies between species and within species and the environmental conditions/stress, however, the most commonly studied are mannitol, proline and glycine betaine (commonly referred to as betaine) [6,7].

The relationships between accumulation of COs and stress tolerance has seen a number of approaches adopted to enhance their concentration [8-12]. These include exogenous application of COs to soil or as foliar sprays [9,11], plant breeding [8] and genetic engineering where the gene responsible for enhanced production of a particular CO is introduced into plants [10,12]. Whatever the approach, there is a need to extract and monitor CO concentrations and hence determine how they affect stress tolerance. There are a variety of methods reported for both the extraction and quantification of these compounds in plants. This review will outline the key methods reported for extraction and analysis of the three most commonly studied: mannitol, proline and betaine. In particular, the review will focus on newer, more efficient methods for their analysis. As there is an extensive range of literature available on the analysis of these compounds, (as their role extends beyond their CO capabilities), this review will focus on the literature where these analytes are investigated in their role as compatible osmolytes.

Extraction of Osmoregulants

Solvent extraction is an essential step prior to the analysis of plant COs. A wide range of solvents have been reported for the extraction of each class of osmolyte (Table 1), in many cases with little justification. For example, a variety of methods have been reported for the extraction of amino acids from plants. Extraction using hot water [13], various

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Analyte	Extracting solvent	Matrix	Ref
Proline	Hot water	Rice	[13]
	3% sulfosalicylic acid	Aspen (*Populus tremula* L.)	[53]
	3% sulfosalicylic acid	Tomato plants	[15]
	3% sulfosalicylic acid	Maize plants	[17]
	3% sulfosalicylic acid	Sugarbeet	[14]
	3% sulfosalicylic acid	Green gram	[16]
	5% sulfosalicylic acid	*Altriplex halimus* L.	[18]
	8% sulfosalicylic acid	Xerophytes and mesophytes	[19]
	10% sulfosalicylic acid	Wheat plants	[20]
	70% methanol	Tomato pollen	[21]
	95% ethanol	Tomato plants	[22]
	MCW (65:25:15)	*Melaleuca* species	[23]
	2% sulfosalicylic acid	Rice leaves	[26]
	3% sulfosalicylic acid	Tomato plants	[25]
	3% sulfosalicylic acid	Mulberry leaves	[27]
	3% sulfosalicylic acid	Sugarbeet	[24]
	3% sulfosalicylic acid	Tomato leaves	[28]
	MCW (15:5:1 v/v/v)	Sugarcane callus culture	[31]
Betaine	80% ethanol	*Enterococcus feacalis*	[34]
	Water	Higher plants	[35]
	Water	*Altriplex halimus* L	[18]
	Methanol	*Zea mays*	[38]
	Methanol	Thai jasmine rice	[37]
	Methanol:anhydrous acetonitrile (1:9)	Green gram	[16]
	MCW (70:20:10, v/v/v)	Barely plants	[42]
	MCW (12:5:1, v/v/v)	*Suaeda maritima* shoots	[39]
	MCW (10:5:6, v/v/v)	*Zea mays* L	[45]
	MCW (12:5:1, v/v)	*Limonium* species and other halophytes	[46]
	MCW (12:5:1, v/v)	Cereals and other grasses	[41]
	MCW (12:5:1, v/v)	Tobacco plants	[44]
	MCW (12:5:1, v/v)	Barley leaves	[40]
	Ethanol	Rape leaf	[33]
	Water	Spinach leaves	[58]
Mannitol	Hot water	*Ligustrum lucidum* Ait	[52]
	80% ethanol	*Phaselous vulgaris* leaves	[54]
	80% ethanol	Celery	[55]
Sugars	MCW (12:5:3)	Ligneous plants	[49]
Sugars	80% ethanol	Muskmelon Fruit	[142]
Sugars and sugar alcohols	80% ethanol	Corn Kernels	[48]
Sugars and sugar alcohols	80% ethanol	Celery Petioles	[55]
Sugars and sugar alcohols	80% ethanol	Aspen (*Populus tremula* L.)	[53]
Proline, betaine	70% ethanol	Bacterial strains	[59]
Proline, betaine	MCN (60:25:15, v/v/v)	Oak leaves	[32]
Proline, betaine	Ethanol: water (80:20)	Spinach leaves	[58]
Proline, betaine, mannitol	MCW (65:25:15, v/v/v)	Peanut and cotton	[61]

Table 1: Extracting solvents reported for the extraction of proline, mannitol and betaine.

concentrations of aqueous sulfosalicylic acid, including 3% [14-17], 5% [18], 8% [19] and 10% [20], 70% boiling methanol [21], 95% ethanol [22] and a mixture of methanol: chloroform: water (65: 25: 15) [23] have all been reported. Aqueous sulfosalicylic acid [19,24-29] and a solvent system consisting of various compositions of methanol: chloroform: water [30-32] have been most commonly used for proline extraction. For quaternary ammonium compounds, and particularly betaine, 80% ethanol, [33,34] water [18,35], methanol: chloroform: water mixtures [36], methanol: acetonitrile (1:9) [16] and methanol [37,38] have all been reported. However, different compositions of methanol: chloroform: water [39-45]; and water [27,40,46,47] have been most frequently described for betaines. Similarly, sugars and sugar alcohols have been extracted using boiling 80% ethanol [48], methanol: water: chloroform (1:1:0.6) [49], methanol: water (1:1) [50] and hot water [51,52]. However, aqueous ethanol is the most common extracting solvent system used for mannitol [48,53-56].

In many studies different extracting solvents have been used to individually extract each CO from the plant of interest [21,53,57,58]. For example, Martino *et al.* extracted proline, along with other amino acids, using an ethanol and water mixture (80:20 v/v), and betaine using distilled water to study the effect of salt stress on the accumulation of these compounds in spinach [58]. Jouve *et al.* used 3% sulfosalicylic acid to extract proline and then in a separate extraction process used 80% ethanol to extract mannitol along with other sugars from *Populus tremula* where these analytes were studied as markers for improvement in stress resistance. The individual extraction of COs from the same plant is time consuming, requires larger samples and solvent volumes, is labour intensive and expensive [53]. There are, however, some examples of concurrent extraction of COs. Simultaneous extraction of proline and betaine, using aqueous ethanol, has been reported [59]. Similarly, a mixture of methanol: chloroform: water (65:25:15) has been used for simultaneous extraction of proline and betaine from oak leaves [32]. Likewise, the combined extraction of amino acids including proline, and betaines has been demonstrated using methanol: water (80:20) [58,60]. The report by Naidu is one of the few examples of the combined extraction of proline, mannitol and betaine. Here, a methanol: chloroform: water (65: 25:15) solvent system was employed [61]. Importantly, none of these studies provided justification or experimental data to support the solvent extraction of choice.

There have been some investigations to determine optimal extraction of COs. For instance, Bessieres *et al.* investigated the best extracting solvent for betaine by comparing cold water, ethanol:chloroform:water (12:5:3) and ethanol: water (9:1). Using cold water, ethanol: chloroform: water (12:5:3) and ethanol: water (9:1), extracted 93.4 ± 4.7, 110 ± 6.0, 105.2 ± 8.6 mmol/g of betaine respectively from *Suaeda maritime*. They concluded that water was the most efficacious for extraction, being inexpensive and almost as efficient as the other extraction systems tested [62]. Similarly, Nishimura *et al.* compared three solvent systems including hot water, 80% ethanol and a mixture of methanol: chloroform: water (12:5:3) for their ability to extract proline and betaine from higher plants grown under elevated salt concentrations [35]. The extraction of betaine was consistent regardless of solvent, however, proline extraction was optimal in hot water (80°C) with 5.5 mmol/kg of proline extracted from freeze dried wheat samples using hot water in comparison to 4.6 and 5.2 mmol/kg using 80% ethanol and mixed solvent systems. As hot water is optimal for proline and as effective as other solvents for extracting betaine, it can be concluded that hot water is an appropriate solvent for combined extraction of these two analytes. It also has the added advantage of being inexpensive and non-toxic. An investigation of optimal conditions for extraction of mannitol has not been reported but hot water has been used in some studies. For example, extraction of mannitol with distilled water from *Ligustrum lucidum* to investigate the concentration of sugar content at various growth levels has been described [52].

It is clear from the literature that a mixture of methanol: chloroform: water, aqueous ethanol, and water are the extracting systems that have been used most commonly for the extraction of all three COs (Table 1). Additionally, methanol: chloroform: water has been chosen frequently for simultaneous extraction of COs in various studies [61]. However, use of this system for extraction is not recommended because of the hazardous nature of chloroform. Moreover, comparison of water with other extracting systems including; methanol: chloroform: water and aqueous ethanol, has shown that it is optimal solvent for extraction of proline and provides comparable results to other solvents for betaine extraction [35]. In addition, the use of hot water for mannitol extraction has also been reported [52] and, given that sugar and sugar alcohols are polar in nature, they should be readily soluble in hot water. Therefore, a hot water extract for mannitol also seems a sensible choice. Moreover, water provides an environmentally friendly, easily available and low cost choice for the extraction of all three osmoregulants. It can be concluded that hot water can be chosen for the extraction of a particular analyte as well as for simultaneous extraction in a study focusing on more than one CO.

Quantification of the Compatible Osmolytes Proline, Betaine and Mannitol

Proline

Proline is an α-amino acid and is polar in nature. It has a carboxylic acid (-CH_3COOH, pK_{a1}=1.95) functional group which makes it positively charged under acidic conditions and an amino group (-NH_2, pK_{a2}=10.64) which makes it negatively charged under alkaline conditions (Figure 1) [63].

Proline has been extensively analysed using a variety of methods including; colorimetry [64-66], chromatography [67-70] and capillary electrophoresis (CE) [7,35], with colorimetry being the most frequently used [64-66], see Table 2 As proline lacks a color absorbing functional group, it can only be analysed after formation of colored derivatives. Chinard reported that proline, at low pH, forms a red product after reaction with ninhydrin in the presence of glacial acetic acid and phosphoric acid, and this compound could be used to quantify proline [71]. However, other amino acids interfered with the proline's determination and an additional ion-exchange or paper chromatography step was required to remove this interference prior to analysis. Improvements were made to the method to make it more selective for proline but they reduced the applicability of the method for routine and rapid sampling. Bates *et al.* suggested a simplified, more effective method where filtered extracts were reacted with ninhydrin and glacial acetic acid at 100°C for 1 hour and the derivatized proline product was extracted with toluene [64]. While this method was an improvement, as interference from free amino acids was minimised, interference from sugars was an issue. Magne and Larher observed that phosphoric acid in the ninhydrin reagent was responsible for the formation of the green colored complex with sugars, particularly sucrose [72]. They suggested using the ninhydrin reagent without phosphoric acid and the use of dilute acetic acid for the analysis of extracts rich in sucrose. While colorimetric methods suffer from poor sensitivity and selectivity, they are still routinely used as they are quick and require no specialised instrumentation. To obtain better sensitivity and selectivity, chromatographic approaches such as gas chromatography (GC) and high performance liquid chromatography (HPLC) have been adopted for proline determination.

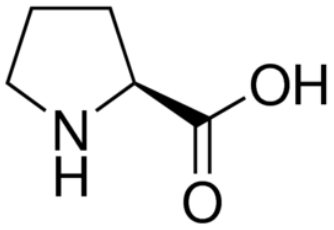

Figure 1: Chemical structure of proline.

GC separates the analytes based on their boiling point and or polarity. The volatilised analytes are transported through the column by an inert gas, typically helium or hydrogen where they are selectively retained by the solid, liquid or polymeric stationary phase which usually coats the inner wall of the separation column [73]. GC's high resolving power makes it ideal for complex samples such as plant extracts. However, while GC is ideal for the analysis of volatile compounds, non-volatile analytes need to be derivatized to make them volatile [74]. Derivatization of functional groups possessing active hydrogens (e.g., -SH, -OH, -NH and -COOH) is of primary importance as they are polar and therefore have reduced volatility [75,76]. The active hydrogen group is usually replaced with a trimethylsilyl group [77] such as trimethylchlorosilane (TMCS) [70], trimethylsilylimidazole (TMSI), N-methyltrimethylsilyltrifluoroacetamide (MSTFA) [68,70], and/or N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) [67]. The derivatives are less polar and sufficiently volatile to allow their elution from the separation column at temperatures that do not cause thermal decomposition of the analyte.

GC can be coupled to a mass spectrometer (MS) for sensitive identification of analytes. GC-MS has been employed for the analysis of proline [67,68,70,78] after derivatisation with a range of derivatising agents. For instance, GC analysis of proline along with 150 other metabolites in potato tubers was achieved after derivatization with a mixture of MSTFA and TMCS; mass spectrometry (MS) was employed for detection [70]. Similarly, determination of proline in grapes, for estimating water and salt stress, was achieved after derivatization with MSTFA [68]. GC-MS using MTBSTFA to derivatize proline was employed to study the performance of alfalfa plants exposed to water stress [67].

In HPLC, analytes generally partition between two liquid phases, the stationary and the mobile. The nature of the stationary phase determines the mechanism of separation. A non-polar stationary phase is ideal for the separation of non-polar analytes while an ion exchanger as the stationary phase is suitable for the separation of charged analytes including amino acids. Reversed phase (RP) HPLC, using a non-polar stationary phase and a polar mobile phase is the most commonly used system. While it is best suited for non-polar analytes, retention of polar analytes such as amino acids is possible by adding an ion pairing reagent (IPR) to the mobile phase. The IPR forms an ion pair with the polar analyte reducing its polarity and enhancing its interaction with the non-polar stationary phase [79] Trifluoro acetic acid (TFA) [80,81], sodium perchlorate [81] and pentadecafluorooctanoic acid [82] are some examples of IPR.

HPLC has been used extensively for the analysis of proline in plants and the methods described vary in terms of separation mechanism and detection mode [6,61,83,84]. As proline is a polar analyte, separation is often achieved on an ion exchange column. For example, Naidu analysed proline in peanut and cotton plants exposed to water stress using a cation exchange column and UV detection at low wavelength. However, as UV detection lacks sensitivity for proline (1.0×10^{-3} µM reported in Naidu's work) [61], a more sensitive approach such as

Analyte	Analytical method	Comments	Matrix	LOD	Ref
Proline	Colorimetry	Coloring reagent-acid ninhydrin	*Atriplex halimus* L.		[18]
	Colorimetry	Coloring reagent-isatin	Grape juice and wine		[65]
	Colorimetry	Coloring reagent-acid ninhydrin	Soybean and sorghum		[64]
	GCMS	MSTFA	Grapes		[143]
	GCMS	MTBSTFA	Alfalfa plants		[67]
	HPLC-UV	C_{18}, dansylated derivatives	*Sorghum bicolour*		[83]
	RP-HPLC-UV	245 nm, phenylthiocarbamyl derivatives,	Alfalfa		[144]
	HPLC-UV	Ninhydrin derivatives	Tomato pollen		[21]
	HPLC-UV	FMOC	Grape juice and wine		[65]
	HPLC-UV	FMOC	Pantoea agglomerans		[85]
	HPLC-UV	OPA	Spinach leaves		[58]
	HPLC-MS	Ligand exchange chromatography, electrospray Ionisation ms	Oak leaves		[32]
	CE-MS		*Arabidopsis thaliana*		[96]
Betaine	Colorimetry	Dragendorff'reagent for visualisation,TLC,PC for removal of interferences	Halophytes		[99]
	Colorimetry	Ammonium reineckates	Sugar beet		[103]
	Thin layer electrophoresis+scanning reflectance densitometry	Plates sprayed with dragendorff reagent	*Suaeda* maritima		[39]
	GC-pyrolysis	FID detection	Cereals and other grasses		[41]
	GC-pyrolysis	FID detection	Barley plants		[106]
	HPLC-UV	Ion exchange column, diode array spectrophotometer set at 195 nm	Spinach		[58]
	HPLC-UV	4-isophenyl trifoliate, silica column	Green gram		[16]
	HPLC-UV	Reverse phase column	*Altriplex halimus* L		[18]
	HPLC-ELSD	Hilic column	*Fructus lycii*		[97]
	CE-UV	CZE mode, 195 nm	Eighteen different species		[35]
	CZE-UV	p-bromophenacyl esters	Higher plants		[114]
	NMR Spectroscopy		*Arabidopsis thaliana*		[96]
	NMR Spectroscopy		Barely		[115]
	NMR Spectroscopy		Rice plants		[118]
	NMR Spectroscopy		Tobacco		[9]
	FABMS		Sugarcane and its relatives		[43]
	FABMS		Tobacco		[44]
	FABMS		*Limonium* species		[46]
	FABMS		Higher plants		[104]
	HPLC-RI		*Pantoea agglomerans*		[85]
Mannitol	Colorimetry	Chromotropic acid for coloration	Fungi and green plants		[132]
	HPLC-PAD	Anion exchange chromatography	Yeast		[145]
	HPLC-PAD	Anion exchange chromatography	Tobacco		[134]
	HP anion exchange electrospray MS	Anion exchange chromatography	Poplar leaves		[135]
proline+betaine	HPLC-PAD	Anion exchange column	Oak leaves		[32]
proline+betaine	CE-UV	Low wavelength 195 nm	higher plants		[35]
proline+betaine	HNMR Spectroscopy	Barely leaves			[118]
proline+mannitol	GCMS	Derivatisation with MSTFA and TMCS	Potato tubers		[70]
proline+betaine+mannitol	HPLC-ELSD	RP column	Halophytes		[6]
proline+betaine	CE-UV	Indirect detection	Spinach leaves, beet root		[7]
proline+betaine+mannitol	HPLC-UV	Low wavelength 195 nm	Peanut, *Melaleuca* and cotton		[61]

Table 2: Analytical methods reported for the analysis of proline, betaine and mannitol.

evaporative light scattering (ELS) detection is attractive. Kalsoom *et al.* used HPLC in combination with ELS detection for comparatively sensitive detection of proline in halophytes with detection limits ten times lower (1.08×10^{-4} µM) than that reported by Naidu (Table 3) [6,61].

Poor sensitivity can also be overcome by derivatization which imparts strong UV absorbing properties to the analyte. The derivatised product is usually less polar than proline itself and separation on a RP [83] column such as octadecyl carbon (C_{18}) or an amino column is more suitable [85]. For example, to obtain improved sensitivity, UV detection of proline in alfalfa plants, exposed to extreme saline conditions, was achieved after derivatisation with phenylthiocarbamyl and proline concentrations as low as 22 nmol/g fresh weight were reported. table 3 for a comparison of LOD data between studies. Other derivatizing

	Analytical method	Linear range	LOD	Recovery (%)	Ref
Proline	Colorimetry	0-200 mg/L	-	96-106	[65]
	Colorimetry	0.1-36 µmoles/g fresh wt	-	-	[64]
	HPLC-MS	0.3125-10 mol L^{-1}	0.4 µM (LOQ)	85-122	[32]
	HPLC-ELSD	25-500 ppm	1.08 × 10^{-4} µmol		[6]
	CE-UV	0.1 mM-100 mM	0.1 mM	90-101	[35]
	GCMS			90	[70]
			0.001 µM		[61]
	CE-UV	5-100 mg/L	11.6 µM	85	[7]
Betaine	Colorimetry		NG	90-95	[99]
	Colorimetry	1-5 mg/mL	-	~100	[103]
	Thin layer electrophoresis+scanning reflectance densitometry		10 µg/20 µL	-	[39]
	GC-pyrolysis	20-150 nmol		78.4	[41]
	HPLC-UV		0.0005 µM		[61]
	HPLC-UV	-	-	95	[58]
	HPLC-UV	15-650 µg/mL	15 µg/mL	79.7	[108]
	HPLC-MS	0.3-10 mol L^{-1}	0.3 µM (LOQ)	-	[32]
	HPLC-ELSD	10-250 µg/mL	3 µg/mL	98-102	[97]
	HPLC-ELSD	25-500 ppm	7.81 × 10^{-5} µmol	-	[6]
	CE-UV	0.1 mM-100 mM	0.1 mM	102-110	[35]
	CZE-UV	0.050-5.0 mM	0.010 mM	94	[114]
	CE-UV	5-100 mg/L	28.3 µM	90-92	[7]
Mannitol	GCMS			108	[70]
	HPLC-UV		0.0025 µM		[61]
	HPLC-ELSD	25-500 ppm	1.43 × 10^{-4} µmol		[6]

Table 3: Linear range, LOD and recovery data reported for proline, betaine and mannitol selected analytical methods of proline, betaine and mannitol.

agents suitable for UV detection of proline include: ninhydrin [21], o-phthalaldehyde (OPA) [58] and 9-fluorenyl-methylchloroformate (FMOC) [65,85]. Derivatization has its drawbacks, it is complicated and time consuming and may lead to formation of side products. Derivitization can be avoided by using MS detection. For example, Oufir *et al.* used HPLC in combination with MS detection, to measure proline to investigate the effect of drought stress on oak leaves [32].

Capillary electrophoresis is both an alternative and complementary technique to HPLC. The distinctive features of CE include, less sample and solvent volume required, rapid analysis times and its ability to simultaneously analyse samples of widely varying polarity [86]. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are two of the most commonly used modes of CE. In CZE, separation is based on differences in mobility of the charged analytes under the influence of an applied electric field. The mobility of an analyte depends on the charge to mass ratio i.e., smaller highly charged species are more mobile when compared to larger, minimally charged ions [87]. In MEKC, separation is based on the distribution of solute between the pseudo-stationary phase (micelles) and the running buffer. Neutral analytes migrating with the electroosmotic flow can interact with micelles and experience a decrease in velocity. Generally, the more hydrophobic the analyte, the more it interacts and the later it elutes [88,89]. Therefore, the polar/ionic species move faster than the less polar analytes.

Although a number of CE methods have been reported for proline analysis [90-93], there are few publications that analyse proline as a CO. Nishimura et al. separated proline and other analytes in a number of plant species using CZE and direct UV detection at low wavelength [35]. They experienced poor analyte sensitivity (100 µM), however, this has since been remedied by using indirect detection [7]. In indirect detection, the background electrolyte (BGE) contains a strongly absorbing electrolyte (also known as a probe) carrying the same charge as the analyte. The displacement of the UV absorbing probe by a UV transparent analyte results in a significant decrease in absorbance and a negative peak is detected [94]. Using indirect detection, a 10 fold improvement in detection sensitivity of proline has been observed. This approach has been used for relatively sensitive measurement of proline (LOD=11.6 µM) in spinach and beetroot using a novel probe, sulphanilamide (Figure 2) [7]. CE in combination with MS has also been used for high mass accuracy and efficient resolution of proline [95,96]. For example, Urano *et al.* used CE-MS for separation and detection of proline and other analytes to compare the metabolic profile of wild type and mutant *Arabidopsis* in relation to dehydration [96].

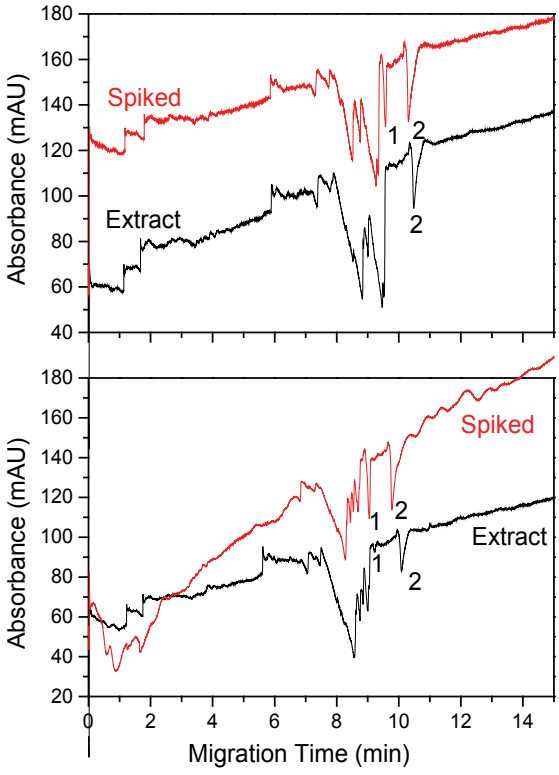

Figure 2: Application of the developed CZE-ID method to spiked and nonspiked extracts from a) beetroot b) spinach. Peak identification; 1. proline 2. betaine. Experimental conditions as reported in Figure 2. Concentration of proline in spiked (51mg/L) and non-spiked (0.9 mg/L) and betaine in spiked (150 mg/L) and non-spiked (104 mg/mL) spinach extract. Concentration of proline in spiked (50 mg/L) and non-spiked (not detected) and betaine in spiked (191 mg/L) and non-spiked (145 mg/mL) beetroot extract. Reprinted from Ref. [7], with permission.

Betaine

Betaine is a zwitterionic compound; it possesses a positive charge at the quaternary ammonium functional group and a negative charge at the carboxylate group (Figure 3) [97]. The pK_a of carboxylic group of betaine is 4.00 [98] which make it possible to develop a positive charge at low pH.

Similar to proline, colorometric analysis of betaine typically relies on removal of interferences by thin layer chromatography, paper chromatography or ion exchange chromatography followed by visualisation of betaine with Dragendorff's reagent [99]. KI-I [100,101] ammonium reineckates [102,103] and phosphotungstic acid [102] have also been used as colorimetric reagents for betaine analysis. However, all of these methods lack sensitivity and are not specific for a particular quaternary ammonium compound. The other limitation is that these methods provide qualitative or semi-quantitative information only. The later drawback can be overcome by using scanning reflectance densitometry in combination with separation techniques [104]. Using this approach, TLC plates sprayed with Dragendorff's reagent are scanned with a spectrophotometer and the reflectance of the background at a particular wavelength (usually yellow or red) is observed. The quenching of red or yellow spots is measured and is used for quantification of betaines [39]. The limitations of these methods have prompted the development of more specific and quantitative approaches for betaine analysis.

Pyrolysis-GC has been repeatedly used for the analysis of betaines [105,106]. It provides a quick and powerful tool for analysing complex and non-volatile samples without the need for derivatization [107]. In pyrolysis, large molecules are thermally broken down into small fragments which are then identified and quantified by GC. For example, accumulation of betaine in [41] cereals and grasses after exposure to water stress has been reported after pyrolysis. The detection was achieved using a flame ionisation detector (FID) [41]. This method has also been used by Ladyman *et al.* for studying water deficit on the distribution and metabolism of betaine in barley [106].

HPLC provides selective and quantitative information and a number of methods have been reported for betaine. As betaine is charged at low pH, ion exchange columns [58,62,108] are commonly used for its separation. However, the use of an RP column [36] has also been reported where retention is increased by derivatization or the addition of an IPR to the mobile phase. For detection, UV is most commonly used [62,108-111] which is most likely due to ease of access to this detector. However, use of RI [36] and MS [112] has also been described for betaine analysis. As betaine lacks a choromophore, UV detection is only possible at low wavelengths [58,62,108] and with relatively high LOD (e.g., 15 µg/mL or 0.128 µM) [108], for sensitive detection derivatization is essential (Table 3) [109,111]. Betaine and its analogues were determined in vegetables after dertivatization with 2-naphthacyl trifluoromethane sulfonate for UV detection, and separation was performed using a RP column [111] achieving detection limits as low as 1 µg/g or 0.0085 µM. Additionally, 4-bromo-phenacyl triflate [109] and 4-isophenyl trifoliate [16] have also been used for derivatization of betaine. The complicated derivatization procedures can be avoided by using ELS detection but with some loss in sensitivity; Shin *et al.* developed a method for the separation of betaine using an HILIC column and employed ELS detection (LOD=3µg/mL) for analysis of *Fructus lycii* [97]. Kalsoom *et al.* measured betaine in halophytes using ELS detection and reported LOD of the order of 8 × 10^{-5} µmol/g fresh weight (Figure 4) [6]. There is only one report employing MS detection for the analysis of betaine; Wood *et al.*

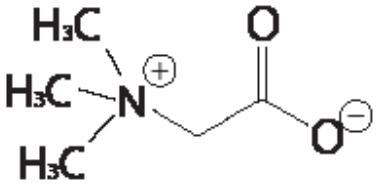

Figure 3: Chemical structure of betaine.

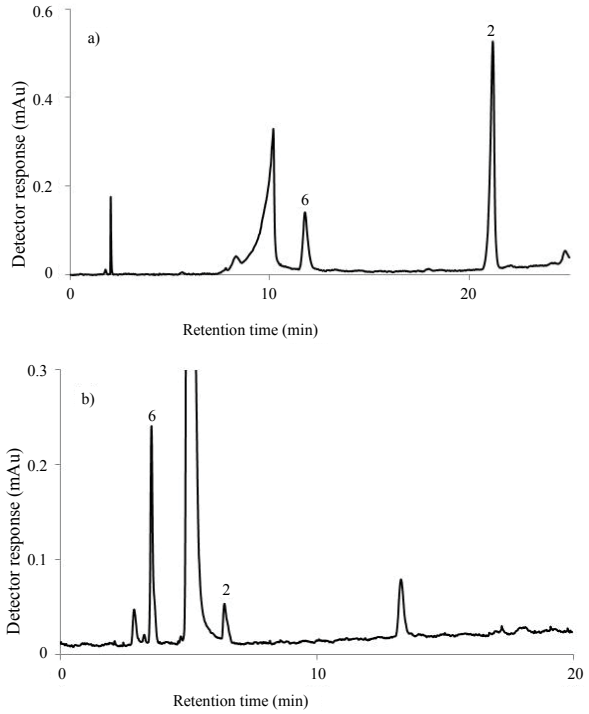

Figure 4: Chromatogram showing separation of osmoregulants in Atriplex cinerea extract using (a) a NH2 column and a mobile phase consisting of 0.1% formic acid and ACN in gradient mode and (b) a C_{18} column and a mobile phase consisting of 0.1% HFBA and ACN in gradient mode. The extract was diluted 3 fold for the C_{18} separation. Peak identification: 2. betaine, 6. sucrose. Reprinted from Ref. [6], with permission.

reported its use for the characterisation of betaines in four different plants [113], however, no LOD data was provided.

Betaines have also been analysed using CE, in both MEKC and CZE modes. Analysis by CZE in combination with UV detection at low wavelength (195 nm) was used to determine betaine in eighteen different plants (e.g., cotton, wheat, barley and alfalfa) [35]. However, poor sensitivity (100 µM) was obtained because of direct UV detection. Derivatization of betaine to form *p*-bromophenacyl esters for more sensitive UV detection (LOD=10 µM) after separation by CZE [114] has also been demonstrated. The ester derivatives, however, are sensitive to pH and thermal changes and therefore Kalsoom *et al.* developed an indirect detection method as an alternative to derivatization for UV analysis (LOD=28.3 µM) [7]. This approach is preferable because it provides a simple and rapid method for simultaneous analysis of proline and betaine without requiring derivatisation.

Another analytical technique, nuclear magnetic resonance (NMR), offers well-resolved, unique and highly predictable spectra for small molecules. In NMR spectroscopy, the magnetic properties of certain

atomic nuclei e.g., ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P are utilised to determine physical and chemical properties of atoms or molecules. There are a number of reports in which NMR spectroscopy has been used for determination of betaine [9,115-118]. For example, accumulation of betaine in wild-type and genetically engineered *Arabidopsis thaliana* was examined using NMR spectroscopy to evaluate the success of the transgenic plants [119]. However, large sample volumes, long run times and poor sensitivity, limits the usefulness of this technique.

Fast atom bombardment mass spectrometry (FABMS) is an ionisation technique that has been used for the determination of chemical structure. In FABMS, the analyte (dissolved in a non-volatile organic phase such as glycerol) is bombarded with a high energy beam of atoms (xenon or argon) to create ions. As a result, a permanent positive charge is created on the analyte by the formation of an adduct ion $[M+H]^+$ with H^+, Na^+ or K^+. These ions are then separated on the basis of charge to mass ratio. This technique has been used for analysis of betaines (Paquet et al.) as a permanent positive charge is created on the zwitterionic form of the analyte by the formation of an adduct with the negative charge of betaine's carboxyl group [104]. Another approach is to derivatize the carboxyl group with an alcohol to form an ester leaving a permanent positive charge on the betaine. This method was used to determine betaine in transgenic tobacco plants [46] and in various species of *Limonium* to investigate its osmoregulatory role [44]. Furthermore, short analysis time [120] and generation of more significant structural information obtained with high-energy collision-induced dissociation FABMS/MS in comparison to other MS/MS methods employing low-energy collision-induced dissociation are the major advantages of this technique [121].

Mannitol

Mannitol is a sugar alcohol and is polar in nature (Figure 5) [122]. Its pK_a value is 13.5 and it can only be negatively charged at high pH [123]. Mannitol analysis has been widely explored for a variety of reasons using a broad range of techniques including photometry [124,125], chromatography [126-128], CE [52,129] and NMR [130].

Similar to proline and betaine, early analysis of mannitol also involved colorimetric methods. For colorimetric analysis, mannitol is oxidised with periodic acid in the presence of formic acid [131] and the formaldehyde produced is estimated by colorimetry after coloration with chromotropic acid [132]. As is typical of other colormetric techniques, it is not specific to mannitol and suffers interference from other sugars.

Another technique, paper chromatography has also been used for the analysis of mannitol [133]. In paper chromatography, mannitol and other sugar alcohols are separated on a paper and are detected by a coloring agent. A variety of coloring agents including *p*-anisidine, perchloric acid, and alkaline periodate-permanganate have all been used for the detection of polyols [133]. However, non-specificity and semi-quantitative analysis are the major limitations of this approach.

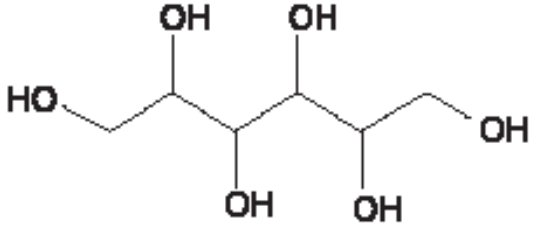

Figure 5: Chemical structure of mannitol.

A limited use of GC-MS has also been reported for analysis of mannitol in its role as an osmoregulant [70]. As previously mentioned for proline, Roessner, *et al.* analysed 150 analytes in potato tubers, which also included mannitol, using GC-MS [70].

As mannitol is a polar molecule and lacks a fluorescent or UV absorbing group, HPLC analysis usually involves separation under alkaline conditions using anion exchange chromatography in combination with pulsed amperometric detection (PAD) [134]. Improved sensitivity can be achieved using MS detection. For example, sensitive determination of mannitol in poplar leaves grown under drought was achieved by PAD followed by MS detection [135]. However, even though PAD provided sensitive detection, co-elution with matrix interferences was an issue for plant samples. Combining MS with PAD provides a more sensitive and selective determination of mannitol along with other carbohydrates in plant extracts. As mannitol and other carbohydrates are negatively charged under highly alkaline conditions, separation can be achieved using an anion exchange column [135].

CE has also been used for the analysis of mannitol, though less frequently. The effect of salt stress in *Kandelia candel* was estimated using CE; mannitol was separated using CZE after complexation with borate and indirect mode was used for detection [136].

For many of the studies presented here, the osmoregulants were for the most part isolated and measured independently [18,27,58,59,85,137-141]. For example, Canamas *et al.* determined proline levels in plant tissues by using RP-HPLC with fluorescence detection [85] and also analysed betaine extracted from the same plant with an HPLC system fitted with a RI detector. Similarly, Hassine *et al.* determined betaine by RP-HPLC in combination with UV detection and proline by a colorimetric method when both osmoregulants were extracted from the same plant [18]. The cost and time associated with completing independent experiments for osmoregulants isolated from the same plant has prompted the development of simultaneous methods for the analysis of the three most commonly explored osmoregulants.

Simultaneous Determination of Compatible Osmolytes

Some attempts have been made to quantify osmoregulants simultaneously from plant extracts. Jones *et al.* estimated betaines and proline in barley leaves grown under water deficit conditions using ^{1}HNMR techniques. While this method was sensitive for betaines (LOD ≈100 µg or 0.1 µM), it was not suitable for accurate determination of proline (LOD ≈50 µg or 0.5 µM), particularly at low levels [116]. Oufir *et al.* used HPLC to determine proline, its analogues and betaine in oak leaves with photodiode array (PDA) detection and an anion exchange column for separation. However, the sensitivity achieved with PDA was insufficient and only proline (LOD=2 µM) and hydroxyproline were detected. The same researchers successfully separated proline (LOQ=0.4 µM), betaine (LOQ=0.3 µM) and its analogues using a size exclusion column for separation and MS for detection [32], however, a long run time (55 min) limited the usefulness of this method. GC-MS has also been used for the simultaneous analysis of 150 analytes (including proline and mannitol) in potato tubers [70] and because MS detection was employed, full separation of the analytes was not necessary.

Naidu determined sugars, sugar alcohols, proline, its analogues and betaines simultaneously in peanut and cotton plants using HPLC coupled to a UV detector [61]. As detection was achieved at

low wavelength the sensitivity of the UV transparent COs was poor (Table 3). The lack of sensitivity was addressed by Kalsoom et al. by developing a HPLC-ELSD method for simultaneous analysis of three osmoregulants i.e., proline, betaine and mannitol (Table 3). In this case the requirement for a relatively volatile mobile phase negated the use of an ion-exchange column. A C_{18} non-polar column and the inclusion of an ion pairing reagent in the buffer to enhance the retention of the polar COs successfully separated the analytes prior to analysis by ELSD. This approach was successful in simultaneously measuring proline, mannitol and betaine in two species of halophytes (*Atriplex cinerea* and *Rhagodia baccata*) and one glycophyte (*Stylosanthes guianensis*) [6].

CE in combination with UV has also been used for simultaneous analysis of COs. For example Nishimura *et al*. determined proline and betaine simultaneously using UV detection at low wavelength (190 nm) and at low pH [35]. However, the sensitivity of this method was poor as direct UV detection was employed. This poor sensitivity can be improved by using indirect detection at 214 nm at low pH (3) [7].

The simultaneous analysis of three osmoregulants by CE is challenging. At any given pH it is not possible to develop a charge on all three osmoregulants. For instance, at low pH proline and betaine carry a positive charge and can be separated by CZE but mannitol remains neutral and elutes unresolved from other neutral analytes. Similarly, at high pH, proline and mannitol can be resolved in their anionic forms but mannitol remains neutral and again elutes with other neutral analytes unresolved and hence cannot be identified. However, an alternative detector, MS, can be used for further identification of analytes. As MS detection is based on the molecular mass of the analytes and as each analyte has different mass it can be readily identified [70]. Furthermore, using MS detection, it should be possible to identify mannitol from other analytes on the basis of molecular mass even if it remains unresolved, thus making the simultaneous analysis of three osmoregulants possible. Therefore, there is need for development of methods using CE in combination with MS to provide sensitive and selective methods for simultaneous analysis of osmoregulants.

Conclusion

It can be concluded that the choice of a method for analysis of a particular osmoregulant is purely dependent on the purpose of work i.e., whether qualitative or quantitative information is required. For example colorimetric methods can be sufficient if only qualitative information for a set of analytes, such as amino acids, sugars and sugar alcohols, and quaternary ammonium compounds, is of interest. However, for more accurate, quantitative analysis of a specific CO, more selective methods such as GC, HPLC, and CE are required.

The other major conclusion from this review is that the individual extraction and quantification of COs, when two or more analytes are studied, is time consuming and labour intensive. Simultaneous extraction of all three key COs (mannitol, proline and betaine) is, therefore, most desirable. This is possible using a number of solvents, one of which is hot water. Similarly, for analysis of COs, colorometric methods are still commonly used to determine each of the COs individually. However, methods for simultaneous determination of COs using various techniques e.g., GC-MS, CE and HPLC in combination with both UV and ELSD detection are also available. A variety of methods for simultaneous analysis of COs available provide a freedom of choice to the user to select a method based on the analytes under study, and sensitivity and selectivity requirements of the analysis. In addition, simultaneous extraction and analysis of COs is fast, simple, requires less solvent for extraction, minimizes waste, is less labour-intensive and inexpensive in comparison to individual extraction and analysis.

For the plant physiologist interested in which CO may be important under certain conditions, simultaneous measurement provides the opportunity to eliminate particular CO from further inclusion in extended work. As many researchers are likely to examine a particular CO because of the techniques/equipment available, the simultaneous measurement of three of the most commonly important COs would allow researchers to focus on a particular CO once others have been shown to be of less importance for particular species or under certain environmental conditions. Having the capacity to analyse all three of the most commonly important CO simultaneously will allow researchers to be more confident that they are targeting the most appropriate CO for a particular species or set of environmental circumstances.

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