Talanta 82 (2010) 118–124

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A new acetonitrile-free mobile phase method for LC–ELSD quantification of fructooligosaccharides in onion (*Allium cepa* L.)

Katherine Downes, Leon A. Terry*

Plant Science Laboratory, Cranfield University, Bedfordshire MK43 0AL, UK

ARTICLE INFO

Article history:
Received 5 October 2009
Received in revised form 23 March 2010
Accepted 7 April 2010
Available online 13 April 2010

Keywords:
Ethanol
Extraction
FOS
Methanol
Sugars

ABSTRACT

Onion soluble non-structural carbohydrates consist of fructose, glucose and sucrose plus fructooligosaccharides (FOS) with degrees of polymerisation (DP) in the range of 3–19. In onion, sugars and FOS are typically separated using liquid chromatography (LC) with acetonitrile (ACN) as a mobile phase. In recent times, however, the production of ACN has diminished due, in part, to the current worldwide economic recession. A study was therefore undertaken, to find an alternative LC method to quantify sugars and FOS from onion without the need for ACN. Two mobile phases were compared; the first taken from a paper by Vågen and Slimestad (2008) [3] using ACN mobile phase, the second, a newly reported method using ethanol (EtOH). The EtOH mobile phase eluted similar concentrations of all FOS compared to the ACN mobile phase. In addition, limit of detection, limit of quantification and relative standard deviation values were sufficiently and consistently lower for all FOS using the EtOH mobile phase. The drawback of the EtOH mobile phase was mainly the inability to separate all individual sugar peaks, yet FOS could be successfully separated. However, using the same onion extract, a previously established LC method based on an isocratic water mobile phase could be used in a second run to separate sugars. Although the ACN mobile phase method is more convenient, in the current economic climate a method based on inexpensive and plentiful ethanol is a valid alternative and could potentially be applied to other fresh produce types.

In addition to the mobile phase solvent, the effect of extraction solvents on sugar and FOS concentration was also investigated. EtOH is still widely used to extract sugars from onion although previous literature has concluded that MeOH is a superior solvent. For this reason, an EtOH-based extraction method was compared with a MeOH-based method to extract both sugars and FOS. The MeOH-based extraction method was more efficacious at extracting sugars and FOS from onion flesh, eluting significantly higher concentrations of glucose, kestose, nystose and DP5–DP8.

1. Introduction

Onion bulbs contain the water soluble carbohydrates fructose, glucose, sucrose and fructans constituting 60–80% of the dry weight [1]. Fructans are oligo- and polysaccharides in which fructosyl units are bound to sucrose by a β-linkage, whereas fructooligosaccharides (FOS) generally only refer to the short chain fructans composed of kestose, nystose and fructofuranosylnystose [2]. FOS are used not only as energy reserves but additionally as osmoregulators due to their solubility in water. The main FOS found in onions are neokestoses which have fructose elongations up to DP19 (degree of polymerisation) from either side of the sucrose unit [3].

The structural and non-structural carbohydrate profile of onion bulbs varies greatly between cultivars [4–6] and throughout storage [5,7]. High fructan concentrations have been associated with increased postharvest storage life potential [8]. In addition, changes in the carbohydrate profile of onion bulbs are important for taste preference as concentrations of fructose and glucose have been positively correlated with likeability and sweetness [9].

Davis et al. [5] investigated the efficacy of different extraction methods for the quantification of sugars and fructans. Three extraction methods were compared and the most efficacious method at extracting sugars and fructans was that described by O'Donoghue et al. [4] with modification. The major differences between these extraction procedures were the solvent used; the O'Donoghue method utilising 62.5% (v/v) methanol (MeOH) whereas the other two methods used aqueous ethanol (EtOH) [10,11]. Due to the higher polarity of the MeOH mixture, fructose, glucose and sucrose tend to be more soluble in MeOH-based solutions than EtOH extraction solvents [5].

* Corresponding author at: Plant Science Laboratory, Cranfield University, Bedfordshire MK43 0AL, UK. Tel.: +44 7500 766 490.
E-mail address: l.a.terry@cranfield.ac.uk (L.A. Terry).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.04.003

Quantification of total fructans can be achieved using an enzyme assay coupled with spectrophotometry [7] or standard liquid chromatography (LC) [2]. However, methods such as LC coupled with an evaporative light scattering detector (ELSD) with aqueous ACN gradient mobile phase [3,10], high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [12] or matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) [5] are capable of quantifying individual FOS of different polymerisation. The LC–ELSD method adopted by Vågen and Slimestad [3] is an excellent method for the fast (10 min) determination of sugars (glucose, fructose and sucrose) and FOS of sizes varying from DP3–DP8 in onion. That said, it requires a large amount of acetonitrile (ACN) which has now become a topical problem.

Recently, a global shortage of ACN caused prices to soar. The reason for this short supply is that unlike MeOH, there are no facilities solely dedicated to the production of ACN, but instead it is sold as a co-product of the plastic, acrylonitrile. Due in part to the global recession, demand for cars and other products which require acrylonitrile plastic has been in decline hence causing a knock-on reduction in ACN production and thus supply.

The aim of this study was therefore to develop a method focused on the use of EtOH rather than ACN to extract and quantify sugars and FOS in onion bulbs. The ACN method used in this study was taken from Vågen and Slimestad [3] who used an EtOH-based extraction method. This said, other papers [5] have found a MeOH-based extraction procedure is more suitable for extraction of sugars from onion. Hence, this study compared two extraction methods; EtOH and MeOH, and two mobile phases; ACN and EtOH to establish a new method for quantification of sugars and FOS from onion without the need to rely on ACN.

2. Materials and methods

2.1. Plant material and sample preparation

Onions cv. Red Baron were grown on peat (Allpress Farms Ltd., Chatteris, Cambs., UK) and harvested on 13th October 2008. Onions were taken for analysis within 2 days of harvest to ensure high fructan content. Three samples were taken from the top, middle and bottom of the field, each of which consisted of four onion bulbs. A longitudinal wedge (5 g) was cut from each of the four bulbs and pooled (20 g) before being snap-frozen in liquid nitrogen and then stored at −40 °C. Frozen tissue was lyophilised using an Alpha 1-4 Christ LDC-1 freeze-dryer (Christ, Lower Saxony, Germany) and pump (Edwards Super Modulo, Sussex, UK) before sugar and FOS analysis.

2.2. Sugar and fructooligosaccharides extraction

Two extraction methods were compared; an EtOH extraction according to Vågen and Slimestad [3] and a MeOH extraction according to O'Donoghue et al. [4], with modifications. Onion powder (150 mg) was added to 2.5 ml 80% (v/v) EtOH and extracted for 30 min at 75 °C using a water bath. The extract was removed using a plastic Pasteur pipette and set aside and then the residue re-extracted using the same method. Again the extract was removed and the pulp re-extracted twice more in 1 ml of LC grade water for 10 min at 75 °C. The extracts were pooled (7 ml) and then passed through a 0.2 μm Millex-GV syringe driven filter (Millipore Corporation, MA, USA).

Similarly onion powder (150 mg), from the same bulb, was also extracted based on the method described by O'Donoghue et al. [4] with modifications [5]. LC grade water (2.25 ml) was added to the onion powder for 10 min at 75 °C to extract the FOS. To the slurry, 3.75 ml MeOH was added to give a final 62.5% (v/v) MeOH solution and extracted for 15 min at 55 °C. The slurry was then passed through a 0.2 μm Millex-GV syringe driven filter. Both groups of extracts were stored at −40 °C until further use.

2.3. LC elution

The two extraction methods were compared for sugars and FOS using a gradient of ACN and water as the mobile phase according to Vågen and Slimestad [3] with slight modifications due to differences in column length. Extracts were thawed and loaded into a LC system (Dionex, CA, USA) with a P680 pump and ASI-100 Automated Sample Injector. The extract (10 μl) was injected into a Prevail Carbohydrate ES column of 250 mm × 4.6 mm diameter, 5 μm particle size (Alltech, UK; Part no. 35101) with a Prevail Carbohydrate ES guard cartridge of 7.5 mm × 4.6 mm diameter (Alltech; Part no. 96435). The mobile phase consisted of LC grade water (A) and ACN (B). The gradient involved a linear increase/decrease of solvent B; 80–50%, 15 min; 50–80%, 5 min; 80% 5 min at a flow rate of 1.0 ml min^{-1} and column temperature held at 30 °C using a STH 585 column oven.

Comparison between mobile phases was only conducted on the samples extracted using the most effective solvent method. The ACN mobile phase method as described above was compared with a new EtOH-based mobile phase using the same LC system and column. The mobile phase consisted of LC grade water (A) and EtOH (B). The gradient involved a linear increase/decrease of solvent B; 85–65%, 9 min; 65–85%, 3 min; 85% 8 min at a flow rate of 0.5 ml min^{-1} and column temperature was set at 40 °C.

2.4. LC quantification

The same detector and standards were used to compare extraction solvents and mobile phases. Eluted carbohydrates were detected using an evaporative light scattering detector (ELSD 2420, Waters, MA, USA) connected to the LC system via a UCI-50 universal chromatography interface. The carbohydrate data was calculated against authentic calibration standards ranging from 0.05 to 4 mg ml^{-1}; fructose, glucose, sucrose, 1-kestose and nystose (Sigma, Dorset, UK) (Fig. 1). The FOS in the range of DP4–DP8 were each calibrated against nystose [3].

2.5. Data analysis

Statistical analyses were conducted using Genstat for Windows Version 9.1.0.147 (VSN International Ltd., Herts., UK). Analysis of variance (ANOVA) was used to identify significant differences in sugar and fructan concentrations measured using each extraction method and each mobile phase. Least significant differences (LSD; $P = 0.05$) were calculated from each analysis. Limit of detection (LOD) was calculated as three times the standard deviation (SD) and limit of quantification (LOQ) as ten times the SD and relative standard deviation (R.S.D. %) was calculated as the (SD × 100)/mean [13]. Standard errors of calibration curve parameters were calculated using SigmaPlot 10.0 (Systat Software, London, UK).

3. Results and discussion

3.1. Extraction procedure

Sugars and FOS were extracted from onion using two different solvents; 80% (v/v) EtOH or 62.5% (v/v) MeOH. The two extracts were only compared using the ACN mobile phase in order to establish the most efficacious extraction method before comparing different mobile phases. Glucose, kestose, nystose, and DP5–DP8

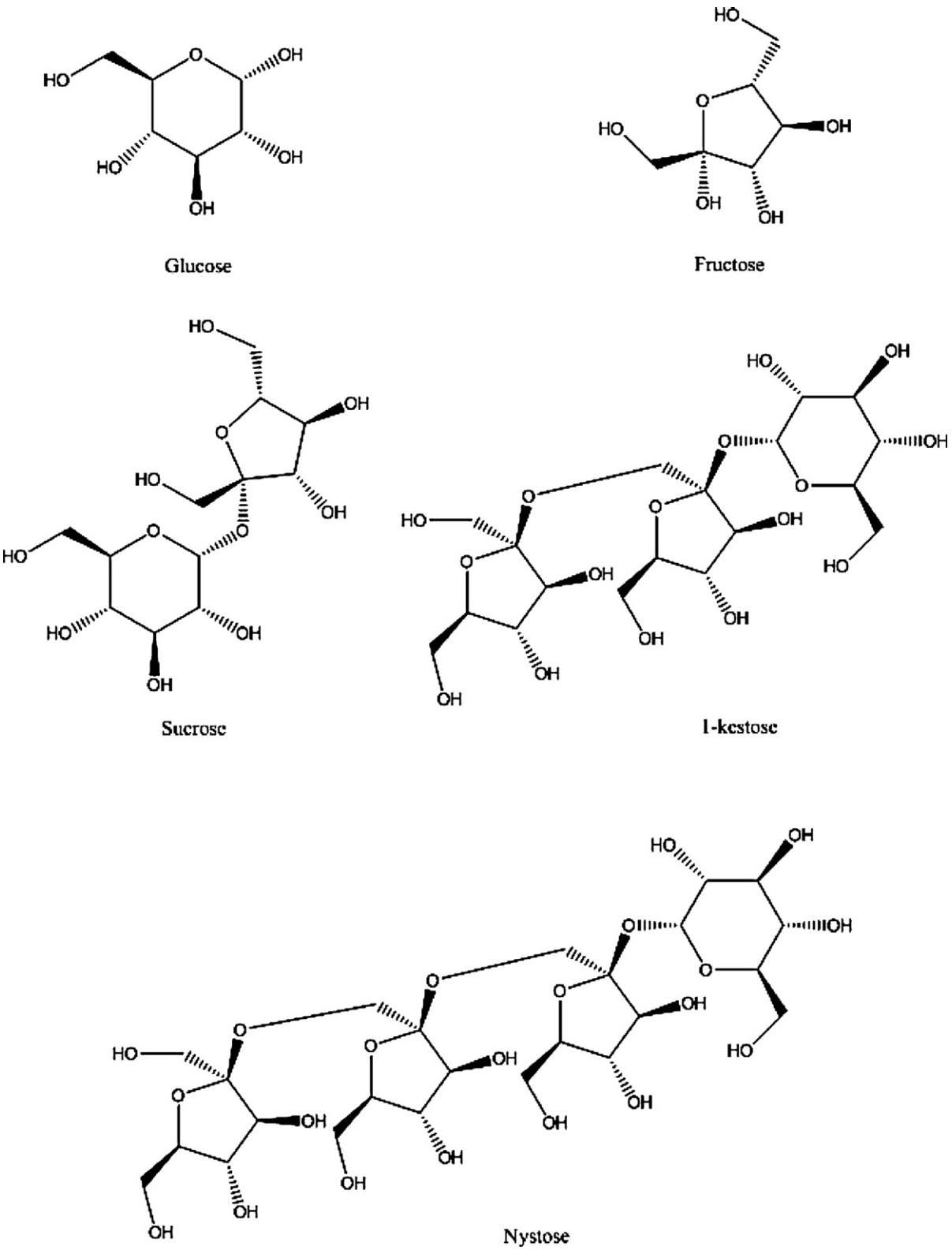

Fig. 1. Structures of glucose, fructose, sucrose, 1-kestose and nystose.

were all significantly higher in samples extracted with MeOH (Fig. 2). Davis et al. [5] compared the 62.5% (v/v) MeOH extraction method used herein with two similar methods to that of Vågen and Slimestad [3]; one using 80% (v/v) EtOH heated for 2 h at 70 °C, although originally it was used on potato [11], and the second using 80% (v/v) EtOH refluxed for 1 h [10]. Higher concentrations of sugars in a variety of onion cultivars and better resolution of fructans were found in samples extracted with the more polar solvent, MeOH, compared with EtOH measured using MALDI-TOF [5]. In addition, the EtOH procedure as described by Vågen and Slimestad [3] took a longer period of time to extract compared with the MeOH extraction. The EtOH extraction required a total of 10 ml

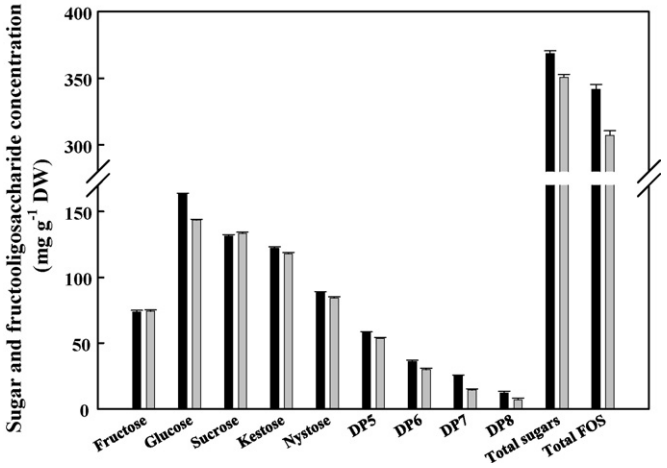

Fig. 2. Mean sugar and fructooligosaccharide concentrations (mg g^{-1} DW) in onion samples extracted with either methanol (MeOH; black bars —) or ethanol (EtOH; grey bars ▬) and quantified using the Vågen and Slimestad [13] acetonitrile mobile phase-based method. Values are means for $n = 9 \pm$ standard error (SE). Total sugars = sum of fructose, glucose and sucrose; total FOS = sum of DP3–DP8.

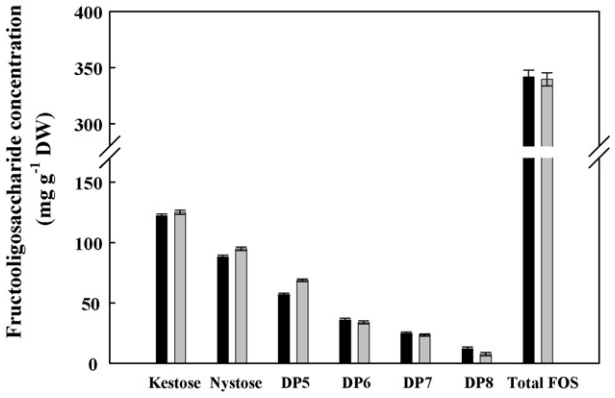

Fig. 3. Mean fructooligosaccharide concentrations (mg g^{-1} DW) of onion samples extracted with methanol and quantified using different mobile phases; acetonitrile (ACN; black bars —) or ethanol (EtOH; grey bars ▬). Values are means for $n = 9 \pm$ SE. Total FOS = sum of DP3–DP8.

EtOH and 1 h 20 min incubation period whereas the MeOH extraction required only 3.25 ml MeOH and 25 min incubation. Therefore, not only did the MeOH extraction elute a higher concentration of FOS and sugars but also required less solvent and a shorter incubation period. Vågen and Slimestad [3] analysed a range of cultivars using fresh onion samples with an EtOH-based extraction solvent. Results herein (Fig. 2) were extracted using the same EtOH-based method and LC conditions but using freeze-dried onion powder. Total sugar concentrations (sum of fructose, glucose and sucrose values) from red onions field cured for 2 weeks plus 4 weeks at 20–25 °C ranged from 5.95 (cv. Reddawn F1) to 6.63 (cv. Red Pearl F1) g 100 g^{-1} FW (*ca.* 595–663 mg g^{-1} DW) [3] whereas total sugar concentrations from freshly harvested freeze-dried red onion powder was 350.7 mg g^{-1} DW (cv. Red Baron) (Fig. 2) and in the range of previous data using freeze-dried onion powder [5,14]. It is therefore worth noting that differences in sugar content between works may be influenced by not only onion growing conditions, age and cultivar but also importantly by sample preparation. The concentration of sucrose in onions cv. Sherpa doubled after 6 weeks curing at 28 °C due to the conversion of FOS into simple sugars [14]. The higher concentration of sugars found by Vågen and Slimestad [3] is therefore most likely due to onion curing as the onions used in this study were freshly harvested to ensure high FOS content but resulted in lower sugar concentrations.

3.2. Elution

Two gradient mobile phases were compared; ACN versus EtOH. The EtOH mobile phase eluted similar concentrations of FOS except for nystose and DP5 which were slightly higher when eluted using EtOH (Fig. 3). However, although the EtOH mobile phase appeared to be as good a method for the quantification of FOS as ACN, it was not possible to resolve all sugar peaks. Two sugar peaks were eluted; the first containing fructose and the second containing both glucose and sucrose (Fig. 4). Modifications to the gradient, column temperature and flow rate would not allow the separation of glucose from sucrose. It was possible to calculate fructose concentrations since the fructose peak was clearly separated (Fig. 4). Fructose is one of the most important sugars in onion due to its organoleptic properties since it is the sweetest of the three sugars [15]. That said, the main drawback of the EtOH mobile phase is that individual sugars cannot be properly determined (Fig. 4); however using the same extract, sugars can be determined in a separate LC run [5]. This run quantifies fructose, glucose and sucrose with a water-based mobile phase under isocratic conditions. Although it would be faster to use the method by Vågen and Slimestad [3], one MeOH extraction and two LC runs adopting EtOH and water mobile phases can acquire the same data in the absence of ACN. Benkeblia et al. [12] used an alternative method of ACN-free LC determination of FOS, however sugars could only be separated using a separate extraction procedure and LC run. When using the ACN mobile phase, an extra peak was observed immediately after the elution of 1-kestose. This peak was not observed by Vågen and Slimestad [3] possibly due to the use of a shorter column. The extra peak was not completely separated from 1-kestose and is therefore most likely a DP3. Other FOS with the same degree of polymerisation includes 6-kestose or neokestose. Shiomi [16] found the major trisaccharides of onions cv. Sapporo-Yellow to be 1-kestose and neokestose therefore the second peak in this study is most likely neokestose. For the purpose of this study the sum of the two peaks was considered as DP3.

3.3. Quantification

Calibration curves should consist of between 5 and 8 values [17], therefore calibration curves of kestose and nystose were generated using solutions of 4, 3, 2, 1, 0.5, 0.1 and 0.05 mg ml^{-1}. From these calibration points a calibration curve based on the 'least squares' methodology should be generated [17], however, typically the calibration curve generated using an ELSD is sigmoidal or exponential [18] including kestose and nystose which both produced second-order polynomial curves. To convert the calibration curves from polynomial to linear, the log 10 of both the peak area and calibration concentrations was calculated [18,19]. The parameters of the second-order polynomial calibration curves using ACN and EtOH as mobile phases were similar for nystose (Fig. 5A and B) however, parameters were almost identical once log-transformed (ACN: $y = 1.4x - 2.5$, EtOH: $y = 1.3x - 2.3$) (Fig. 5C and D). This same trend was found for kestose (Table 1) where even though the calibration curve parameters were close when calculated as a polynomial curve, the log-transformed linear parameters were closer between mobile phases. Fig. 5E and F demonstrate that applying a polynomial curve to the log-transformed calibration curve does not produce particularly accurate parameters and therefore a linear calibration curve applied to the log-transformed data is preferred.

The LOD is the lowest concentration which can be discriminated from noise levels and concentrations which lie between the

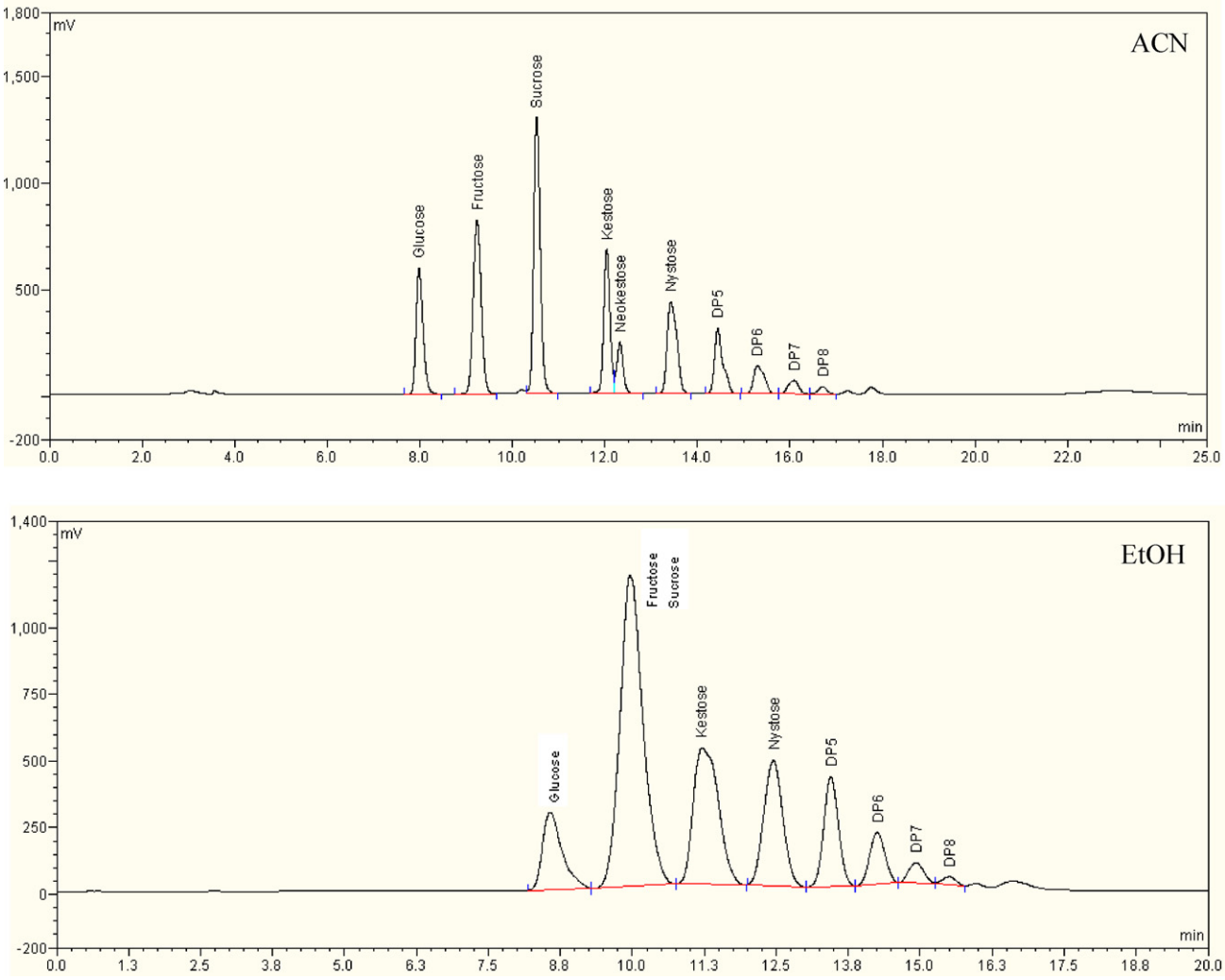

Fig. 4. Chromatographic profile of sugars and fructooligosaccharides found in onion using different mobile phases; acetonitrile (ACN) and ethanol (EtOH).

Table 1
Comparison between the parameters of kestose and nystose calibration curves plus standard errors quantified using two mobile phases; acetonitrile (ACN) and ethanol (EtOH) and calculated as polynomial second-order curves ($y = ax^2 + bx + c$) and as log-transformed linear curves ($y = bx + c$).

	ACN mobile phase	EtOH mobile phase
Kestose		
Non-log polynomial ($mg\,ml^{-1}$)		
a	6.3 ± 1.49	11.8 ± 1.53
b	57.4 ± 5.95	53.1 ± 6.11
c	-6.4 ± 3.95	-5.6 ± 4.06
r^2	0.99	0.99
Log linear ($mg\,l^{-1}$)		
b	1.4 ± 0.01	1.3 ± 0.03
c	-2.5 ± 0.03	-2.3 ± 0.08
r^2	0.99	0.99
Nystose		
Non-log polynomial ($mg\,ml^{-1}$)		
a	6.1 ± 1.24	10.9 ± 1.55
b	52.1 ± 4.94	53.3 ± 6.18
c	-5.6 ± 3.28	-5.6 ± 4.10
r^2	0.99	0.99
Log linear ($mg\,l^{-1}$)		
b	1.4 ± 0.01	1.3 ± 0.03
c	-2.5 ± 0.03	-2.3 ± 0.08
r^2	0.99	0.99

LOQ and the LOD are not accurate but can be described as semi-quantitative [17]. The LOD and LOQ were calculated for both ACN and EtOH mobile phases. Although the LOD and LOQ values were lowest for DP5, DP7 and DP8 when eluted using ACN, the LOD and LOQ values were lower for kestose, nystose and DP6 when eluted using EtOH (Table 2). The LOD and LOQ were more consistent when using the EtOH mobile phase with the values ranging from 1.5–4.6 to 3.7–15.2 $\mu g\,ml^{-1}$, respectively compared to the ACN, LOD and LOQ which ranged from 1.9–9.8 to 6.4–32.7 $\mu g\,ml^{-1}$, respectively. The R.S.D showed greatest variation for DP6 and DP8 using the ACN mobile phase but also for DP7 and DP8 using the EtOH mobile phase; that said, R.S.D. values were all below the recommended level of 20% [20]. The R.S.D. values for the FOS using the newly developed EtOH-based method ranged from 0.39 to 16.30% and hence, indicated good stability under the EtOH mobile phase conditions.

3.4. Current ACN situation

Supplies of ACN are improving; however, prices still remain high in comparison with EtOH and MeOH. If supplies improved sufficiently to reduce the price of ACN, the ACN method could be readopted as the method of choice enabling the quantification of both sugars and FOS in onion tissue in a single LC run. That said,

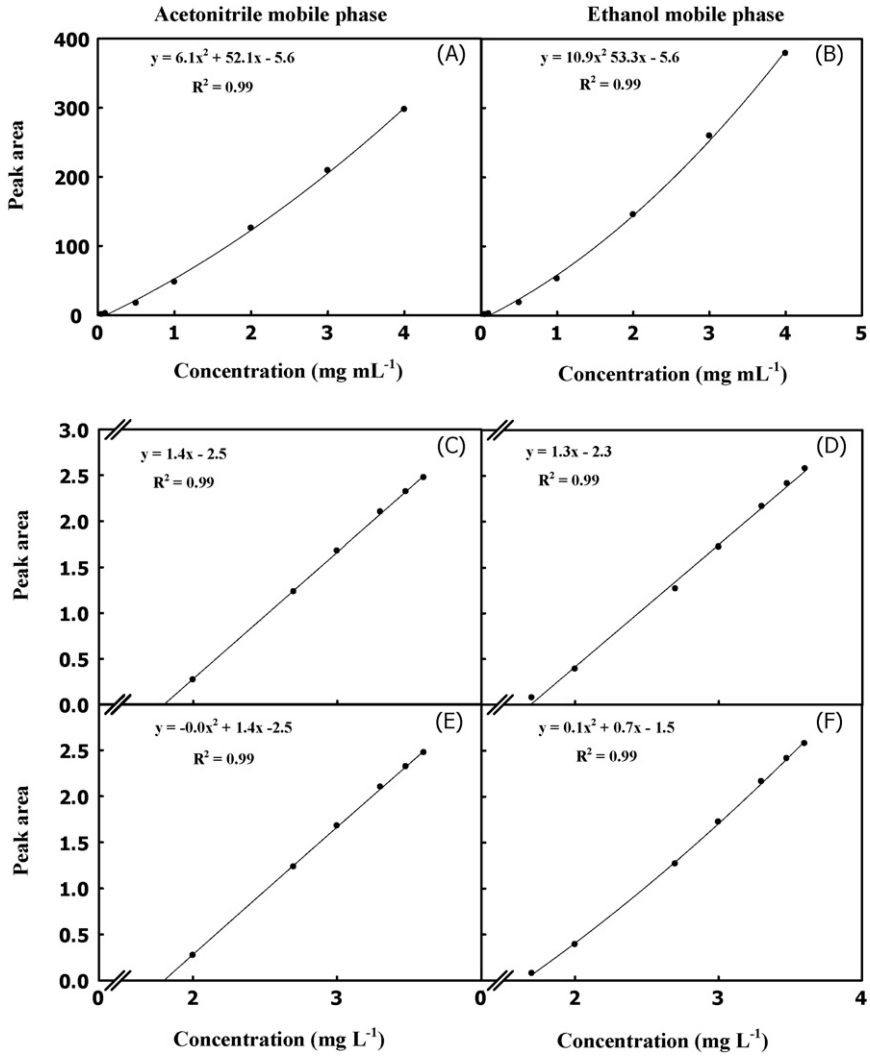

Fig. 5. Calibration curves of nystose quantified using acetonitrile (left) or ethanol (right) as mobile phases. (A and B) Second-order polynomial calibration curves; (C and D) log-transformed linear calibration curves; (E and F) log-transformed second-order polynomial calibration curves.

Table 2
Performance parameters (limit of detection (LOD), limit of quantification (LOQ) and R.S.D (relative standard deviation)) of the different fructooligosaccharides (DP5–DP8) when eluted using different mobile phases; acetonitrile (ACN) or ethanol (EtOH).

Compound	ACN mobile phase			EtOH mobile phase		
	LOD ($\mu g\,ml^{-1}$)	LOQ ($\mu g\,ml^{-1}$)	R.S.D (%)	LOD ($\mu g\,ml^{-1}$)	LOQ ($\mu g\,ml^{-1}$)	R.S.D (%)
Kestose	3.0	10.0	0.58	1.5	4.5	0.39
Nystose	2.6	8.6	0.97	1.8	5.9	0.60
DP5	1.9	6.4	1.03	3.0	10.1	1.39
DP6	9.8	32.7	6.28	4.6	15.2	3.66
DP7	2.6	8.7	3.43	4.5	14.9	7.92
DP8	2.2	7.2	5.09	3.7	3.7	16.30

irrespective of purchase cost, ACN is relatively toxic and more expensive to dispose of appropriately compared with EtOH since ACN requires detoxification [21].

4. Conclusion

In conclusion, MeOH was confirmed as the more efficacious solvent for extracting onion FOS and sugars. In addition, modifications to current methods resulted in a combined MeOH and water extraction to remove both sugars and FOS using one simple procedure. Due to the high cost of ACN following the worldwide shortage, an ACN-free LC method could be considered given that EtOH was found to elute concentrations of FOS in the same range as ACN with consistently low LOD, LOQ and R.S.D values.

Acknowledgements

This work forms part of a larger HortLink project (HL0182; Sustaining UK Fresh Onion Supply by Improving Consumer Acceptability, Quality and Availability) and is financially supported by the UK Government (Department for Environment, Food and Rural Affairs; Defra), Horticultural Development Company (HDC) and UK industry representatives. The authors thank Balwinder Cook and Jordi Giné Bordonaba for technical assistance.

References

[1] B. Darbyshire, R.J. Henry, New Phytol. 81 (1978) 29–34.
[2] L. Jaime, F. Martínez, M.A. Martín-Cabrejas, E. Mollá, F.J. López-Audréu, K.W. Waldron, R.M. Esteban, J. Sci. Food Agric. 81 (2000) 177–182.
[3] I.M. Vågen, R. Slimestad, J. Sci. Food Agric. 88 (2008) 404–411.
[4] E.M. O'Donoghue, S.D. Somerfield, M. Shaw, M. Bendall, D. Hedderly, J. Eason, I. Sims, J. Agric. Food Chem. 52 (2004) 5383–5390.
[5] F. Davis, L.A. Terry, G.A. Chope, C.F.J. Faul, J. Agric. Food Chem. 55 (2007) 4299–4306.
[6] B. Rodríguez Galdón, C. Tascón Rodríguez, E.M. Rodríguez Rodríguez, C. Díaz Romero, J. Food Compos. Anal. 22 (2009) 25–32.
[7] G.A. Chope, L.A. Terry, P.J. White, Postharvest Biol. Technol. 39 (2006) 233–242.
[8] M. Suzuki, J.A. Cutcliffe, Can. J. Plant Sci. 69 (1989) 1327–1333.
[9] L.A. Terry, K.A. Law, K.J. Hipwood, P.H. Bellamy, Symp. Fructic 05: Information and Technology for Sustainable Fruit and Vegetable Production, Montpellier, France, 12–16 September 2005, 2005.
[10] R. Kahane, E. Vialle-Guérin, I. Boukema, D. Tzanoudakis, C. Bellamy, C. Chamaux, C. Kik, Environ. Exp. Bot. 45 (2001) 73–83.
[11] R. Viola, H.V. Davies, Potato Res. 35 (1992) 55–58.
[12] N. Benkeblia, S. Onodera, N. Shiomi, Food Chem. 87 (2004) 377–382.
[13] S. Muñoz, M. Mestres, O. Busto, J. Guasch, Anal. Chim. Acta 628 (2008) 104–110.
[14] K. Downes, G.A. Chope, L.A. Terry, Postharvest Biol. Technol. 55 (2010) 36–44.
[15] J. Hallfrisch, FASEB J. 4 (1990) 2652–2660.
[16] N. Shiomi, J. Fac. Agric. Hokkaido Univ. 58 (1978) 548–556.
[17] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3–10.
[18] B.T. Mathews, P.D. Higginson, R. Lyons, J.C. Mitchell, N.W. Sach, M.J. Snowden, M.R. Taylor, A.G. Wright, Chromatographia 60 (2004) 625–633.
[19] W. Li, J.F. Fitzloff, J. Pharm. Biomed. Anal. 25 (2001) 257–265.
[20] Y. Kazakevich, R. LoBrutto (Eds.), HPLC for Pharmaceutical Scientists, John Wiley & Sons, Inc., Hoboken, NJ, 2007, p. 483.
[21] R.L.V. Ribeiro, C.B.G. Bottoli, K.E. Collins, C.H. Collins, J. Braz. Chem. Soc. 2 (2004) 300–306.