Contents lists available at [ScienceDirect](#)

Food Chemistry

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

Analytical Methods

Hydrophilic interaction chromatography and evaporative light scattering detection for the determination of polar analytes in Belgian endive

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21 1 Abstract

22 Belgian endive (*Cichorium intybus* L. var. *foliosum* Hegi), a popular produce in northern Europe, has been
23 thoroughly studied in regard to its bitter sesquiterpene lactones content. Much less is known about on its
24 sweetness and crunchiness, which are typically linked to the content of polar compounds such as sugars,
25 organic acids and salts. Through HILIC-HPLC-MS, it was shown that simple sugars, amino acids, and potassium
26 chloride are abundant in Belgian endive extracts. Subsequently, a HILIC-HPLC-ELSD method for the analysis of
27 such compounds with run times below six minutes was developed. Recoveries varied between 80 and 110 %
28 and an average reproducibility was 7.5 RSD%. Finally, the method was applied to the study of three difference
29 Belgian endive varieties. Takine, a variety known for its sweet taste, was found to contain significantly higher
30 levels of fructose, and lower levels of potassium and glutamine.

31 2 Keywords

32 *Cichorium intybus*; hydrophilic interaction liquid chromatography; high performance liquid chromatography;
33 evaporative light scattering detector

34 3 Compounds

35 Glucose (PubChem CID: 5793), fructose (PubChem CID: 5984), sucrose (PubChem CID: 5988), glutamine
36 (PubChem CID: 5961), potassium chloride (PubChem CID: 4873)

37 4 Introduction

38 Belgian endive (*Cichorium intybus* L. var. *foliosum* Hegi) is a vegetable popular in northern Europe. Its white
39 etiolated heads (chicons) are served both fresh and cooked (Lucchin, Varotto, Barcaccia, & Parrini, 2008) and
40 are sought after because of their delicate bitter-sweet taste (Lucchin et al., 2008). Its aroma is very weak, with
41 the only relevant odor being a “green” note attributed to hexanal and (E)-2-hexenal (Goetz-Schmidt & Schreier,

1986). The bitter taste is related to the presence of sesquiterpene lactones. They have been extensively studied with regard to their composition (Sessa, Bennett, Lewis, Mansfield, & Beale, 2000; Zinke & Holzer, 1953), relevance to taste (Chadwick, Gawthrop, Michelmores, Wagstaff, & Methven, 2016; Leclercq, 1992) and health effects (Bischoff et al., 2004; Ren, Zhou, & Ye, 2004). Sweetness of Belgian endive and its relation to sugars has been much less studied, although it affects perception of bitterness as well. Other sensory properties such as crunchiness and salty or umami taste and their relation to osmolytes, salts and amino acids have never been investigated in Belgian endive. Interestingly enough, these little-studied compounds are all polar.

Official food composition tables give an insight into the content of the soluble polar fraction (values listed in supporting materials table S1). The most abundant components are simple sugars, at an average of 2 % w/w. Among the minerals, potassium is the most abundant, with measurable amounts of calcium, chlorine and phosphorus. Inorganic cations are known to produce a salty, and, especially for potassium, a bitter taste (Murphy, Cardello, & Brand, 1981; Van Der Klaauw & Smith, 1995). Together with the sugars, they have a role in the osmoregulation of cells, and are therefore linked to the crunchiness of the leaves (Talbot & Zeiger, 1996). Glutamic acid is the most abundant amino acid and induces the umami taste (Schiffman, Sennewald, & Gagnon, 1981). Both potassium and glutamate appear to be present at a concentration above the sensory threshold and might have a synergistic interaction with other taste molecules (Keast & Breslin, 2003). Organic acids were not listed in the tables as they do not constitute important nutrients. However, they are relevant to the sensory properties in general, having often both sour and astringent properties (Lawless, Horne, & Giasi, 1996; Rubico & McDaniel, 1992; Sowalsky & Noble, 1998). Amongst the most abundant organic acids in leafy vegetables are malic, citric, tartaric and quinic acid (Flores, Hellín, & Fenoll, 2012), which are expected in relevant quantities in Belgian endive as well.

Water soluble polar molecules from plant matrixes can be either cation, anions, zwitterions and neutral. A wide array of methods and detectors is traditionally required to cover the full range. Normal phase chromatography with aqueous eluents has long been used for analysis of simple sugars and oligosaccharides (Linden & Lawhead,

1975). Since Alpert minted the name hydrophilic interaction liquid chromatography (HILIC) (Alpert, 1990), the technique has received much attention in the fields of drug analysis and metabolomics. Its use in food analysis, however, is still lagging behind. Between the few applications, notable are the untargeted detection of key tastants in mandarin juice (Charve, Chen, Hegeman, & Reineccius, 2011) and of novel umami compounds in soy sauce (Shiga et al., 2014). HILIC has been used for determination of sugars (Linden & Lawhead, 1975), organic acids (Y. Huang, Tian, Zhang, & Peng, 2012), amino acids, and inorganic salts (Z. Huang et al., 2009). Theoretically it has the potential to analyze all these classes of compounds together, however, to our knowledge no study has implemented this yet.

The need for such integrated analyses comes from a larger study investigating influence of genetic diversity and growing conditions on the sensory and chemical profile of Belgian endive. Therefore, the objective of this study was threefold: 1) to evaluate the range of analytes amenable to HILIC through HPLC-MS, 2) to develop a HPLC-ELSD method for their quantification focused on speed, and 3) to show the applicability of the developed method to real-world Belgian endive samples

5 Materials and methods

5.1 Farming and sample extraction

Belgian endive (cultivars Takine, Symphonie and Vintor) was field-grown and forced at Inagro vzw (Rumbeke-Beitem, Belgium). Roots were harvested from the field on 19/11/2013 and forced at 11 °C from 27/5/2014 to 24/6/2014. After that, heads were separated from the root and kept refrigerated at 4 °C. For each cultivar, random samples were analyzed. After removing the two outer leaves, the remaining leaves were separated from the pit with a knife, immediately frozen in liquid nitrogen and ground to a fine powder using a CryoMill grindomixer MM200 (Retsch, Haan, Germany). Samples were kept in dry ice for transport to the lab, and stored subsequently at -80 °C until analysis. For each sample, 300 mg was weighed in a 2 mL Eppendorf tube, to which 1.4 mL of extraction buffer (water with 1 mL·L⁻¹ of formic acid) was added before thawing. The polar

compounds were extracted under continuous shaking at 30 °C for 15 min in a Thermomixer (Eppendorf, Rotselaar, Belgium), followed by centrifugation at 15000 rpm and 4 °C for another 15 min (Ultrafuge, Eppendorf). Approximately 1 mL of the supernatant was drawn out with an insulin syringe and filtered through a 13 mm x 0.2 µm PTFE disc filter (VWR, Leuven, Belgium) to a glass vial (Phenomenex, Utrecht, Netherlands). The extract was diluted in mobile phase prior to injection to a total volume of 1 mL in a fresh glass vial, with the dilution ratio ranging being 1:20 for the MS experiments and 1:5 for the ELSD. For the MS experiments, EDTA (Sigma Aldrich, Diegem, Belgium) was added to the dilution liquid as to reach a final concentration of 100 ppm in the diluted sample.

5.2 Eluent and buffer preparation

HPLC grade water and acetonitrile were purchased from Biosolve (Valkenswaard, the Netherlands). Ammonium acetate, ammonium formate, ammonium hydrogen carbonate, formic acid, acetic acid, ammonium hydroxide (25 %), and triethylamine were of HPLC or HPLC/MS grade and were purchased from Sigma Aldrich (Diegem, Belgium).

Various eluents were prepared consisting of acetonitrile as the weak solvent, water as the strong solvent, and a modifier. The following modifiers were used: formic acid (0.1 %, pH ≈2.7), ammonium formate (10 mM, pH 3.5), ammonium acetate (10 mM, pH 4.5), ammonium formate (10 mM, pH 6.5), ammonium acetate (10 mM, pH 6.8), ammonium bicarbonate (10 mM, pH 7.7), ammonium formate (10 mM, pH 9.5), ammonia (0.1%, pH ≈10.7), and triethylamine (0.1 %, pH ≈11.5). The molarities refers to the sum of the concentrations of the acid and its conjugate base in the eluent, and the percentages to the gravimetric ratio (kg·kg⁻¹). Calculations of the weights were made with the Buffer Maker software (ChemBuddy, Marki, Poland). Eluents were prepared in a temperature controlled room (24 °C). Water, acetonitrile, ammonium acetate, ammonium formate, and ammonium hydrogen carbonate were measured gravimetrically assuming a density of 1 kg·L⁻¹ for water and 0.76 kg·L⁻¹ for acetonitrile (Moreau & Douhéret, 1975). The volumes of the other liquids were obtained from the weight using the following densities (Lide & Haynes, 2009; O'Neil, 2006): ammonium hydroxide 0.900 kg·L⁻¹

113 ¹, triethylamine 0.726 kg·L⁻¹, formic acid 1.220 kg·L⁻¹, acetic acid 1.045 kg·L⁻¹. All components were directly
114 mixed in the eluent without further pH checking: reproducibility was verified by the stable retention times over
115 different eluent batches. It must be noted that anyway, it would not be possible to measure with conventional
116 means the pH of solutions with a high content of organic solvents (Suu et al., 2015). Solvents were premixed at
117 the starting and final eluting ratio together with the modifiers, which were kept of constant concentration
118 throughout the run.

119 5.3 HPLC-MS analysis

120 HPLC-MS separation was performed using a modified Alliance 2695 system (Waters, Zellik, Belgium). The
121 following modifications were performed on the Alliance separation module: 1) all the internal tubing was
122 replaced with custom cut, burr-free electropolished stainless steel tubes of 0.254 mm I.D. (Valco Vici,
123 Schenk, Switzerland); with the only exception being the tube leading to the primary pump head from the
124 gradient valve (0.508 mm I.D.), 2) the eluent filter placed after the pumps was removed, 3) the original sample
125 loop was replaced with a 30 µL PEEK-lined stainless steel tubing (IDEX Health & Science, Wertheim-Mondfeld,
126 Germany), 4) the sample needle was replaced with a titanium nitride-coated needle (Fischer analytics GMBH,
127 Weiler bei Bingen, Germany), 5) a 25 µL syringe (Hamilton, Bonaduz, Switzerland) was installed, and 6) a 100
128 µm I.D. PEEK tubing was placed between injector and column, and between column and detector.

129 Four column were tested: 1) Nucleodur HILIC 150 x 2.1 mm x 3.5 µm (Macherey Nagel, Düren, Germany), 2)
130 ZIC-HILIC 150 x 2.1 mm x 3 µm (Merck Millipore, Billerica, MA), 3) Luna diol 150 x 2.1 mm x 3.5 µm (Phenomenex),
131 and 4) Xbridge amide 150 x 2.1 mm x 2.5 µm (Waters, Zellik, Belgium). Every column was coupled to an
132 adequate precolumn of the same diameter and stationary phase. A linear gradient, 20 min long, spanning from
133 90 % to 50 % acetonitrile was used for every column, except the diol column which started at 80 %. The column
134 oven was always kept at 35 °C. The injection volume was 1 µL.

135 Detection was performed on a standard QTOF Micro (Micromass). The following source parameters were used
136 in negative electrospray mode: capillary voltage: 2500 V, sample cone: 25 V, extraction cone 1 V, desolvation
137 temperature: 250 °C, desolvation flow: 400 L·h⁻¹, no cone flow, source temperature: 110 °C, collision energy: 3
138 V. Acquisition parameters were as follows: scan time: 0.6 s, inter-scan time: 0.1 s, reference scan (leu-
139 enkephalin): 60 s, centroid mode, centroid threshold: 1, minimum point: 5, Np factor: 0.97. The mass
140 spectrometer was tuned to a resolution of 5500. Its performance was monitored daily by calibrating with a
141 solution of sodium formate prepared according to the manufacturer's specifications, and ensuring that the
142 residuals of the polynomial calibration were under 5 mDa. Deadtime correction (Np factor) was calculated by
143 injecting increasing concentrations of leu-enkephalin and empirically measuring the correct value.

144 5.4 Identification of peaks

145 First, chromatograms were manually inspected for peaks above baseline noise, and the putative
146 quasimolecular ion and retention time annotated in a table. Entries present in the blank samples or exclusively
147 in two column-and-eluent combinations were considered as ghost peaks and removed from the table. The
148 filtered accurate masses were matched using a window of 30 ppm against three sources: 1) an in-house list
149 containing the hydrophilic molecules expected to be present in the matrix, 2) the list compiled by Abu-Reidah
150 et al. (Abu-Reidah, Contreras, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013), and 3) the
151 Metlin database (Smith et al., 2005). Loss of hydrogen, formation of adducts with acids or metals, and loss of
152 water or CO₂ were considered when comparing the putative quasimolecular ions. For positive matches of
153 quasimolecular ions, fragments from in-source dissociation were matched whenever MS/MS data was available
154 from literature.

155 5.5 HPLC-ELSD analysis

156 HPLC-ELSD was performed on a modified Agilent 1100 system composed of the following modules: G1322A
157 degasser, G1311A quaternary pump, G1329 autosampler with G1330B thermal module, G1316A oven, and
158 G4260B ELSD detector. The following modifications were applied: all the factory installed tubing after the first

159 pump head was replaced with custom cut, burr-free electropolished stainless steel tubing from Valco (0.254
160 mm I.D.), a new stainless steel sample loop with a capacity of 30 μL was installed, and the oven preheater was
161 bypassed. In order to ensure maximum thermal homogeneity for the column and the eluent without the
162 standard preheater, a custom column holder was built from an L-shaped aluminum extruded bar, covered with
163 copper thermal tape. The holder was placed between the two oven-assemblies, the column placed inside the
164 holder, and insulating foam covered the gap between the two oven assemblies at the back, and the space
165 between the column holder and the plastic cover. Extra copper tape was glued around the tubing in the oven
166 just before the inlet of the column to form a simple heat exchanger. Column outlet and detector were
167 connected by means of a 0.178 mm I.D. PEEK tubing.

168 Analysis was performed on an Xbridge amide column (100 mm length x 3 mm I.D. x 1.7 μm particle size)
169 coupled to a cartridge guard column of the same format and stationary phase. The optimized chromatographic
170 conditions were as follows: isocratic acetonitrile/water 75/25 with ammonium acetate 3 mM, 5 μL injection,
171 oven temperature 80 $^{\circ}\text{C}$, run time 7 min. The parameters for the ELSD were as follows: evaporator
172 temperature: 90 $^{\circ}\text{C}$, nebulizer temperature: 50 $^{\circ}\text{C}$, gas flow: 1.2 SLM, data rate: 40 Hz, LED intensity: 100 %,
173 PMT gain: 1, smooth: 10. Pressure drop was calculated according to the formula:

$$\Delta p \text{ (MPa)} = 1.724 \frac{L \cdot \eta \cdot F}{d_p^2 \cdot d_c^2}$$

174 Where L is the length of the column in mm, η the viscosity of the mobile phase (cP), F the flow rate in $\text{mL} \cdot \text{min}^{-1}$,
175 d_p the particle diameter in μm and d_c the internal diameter of the column in mm (Thermo Fisher Scientific,
176 2015).

177 5.6 Calibration and performance of the HPLC-ELSD method

178 Standards of glucose, fructose, sucrose, glutamine and potassium chloride were diluted in extraction buffer in
179 concentrations ranging from 10 to 0.001 $\text{g} \cdot \text{L}^{-1}$. For the salts, the amount of individual ions in solution was
180 calculated as follows:

$$C_I = \frac{C_s \cdot MW_I}{MW_S}$$

181 Where C_I is the concentration of the ion of interest in solution and C_s is the concentration of the salt in $\text{g}\cdot\text{L}^{-1}$,
 182 MW_I is the molecular weight of the ion and MW_S the molecular weight of the salt in u. With a pipette 300 μL
 183 were mixed in an Eppendorf tube with 1400 μL of extraction buffer, reaching the same final volume as when
 184 extracting endive. Assuming a density of the plant material of $1 \text{ kg}\cdot\text{L}^{-1}$, the concentrations of the diluted stock
 185 solutions used in this step are equivalent to those found in the plant in fresh weight. Three technical replicates
 186 for each dilution level were processed and injected in the HPLC. An inverse linear model (Tellinghuisen, 2000)
 187 was built for each analyte by fitting the log of the concentration over the log of the normalized area:

$$\log(C_{\text{analyte}}) = a + b \cdot \frac{\log(A_{\text{analyte}})}{\log(A_{I.S.})}$$

188 Where A_{analyte} and $A_{I.S.}$ are the areas of the analyte and internal standard respectively, a is the intercept, b the
 189 response factor, and C_{analyte} the concentration of the analyte in solution.

190 The recoveries were determined by the spike method. A sample was found with concentration of analytes
 191 close to the average values, which was then spiked separately two times for each analyte. Recoveries of
 192 individual analytes were calculated with the following formula:

$$\text{Recovery} = \frac{C_{\text{sample}} + C_{\text{added}}}{C_{\text{measured}}} * 100$$

193 The limit of detection was defined as the concentration at which the height of a peak was 3 times the height of
 194 the noise level recorded in a clean part of the chromatogram. Intra-day reproducibility was defined as the
 195 percent relative standard deviation (RSD %) measured for five technical replicates (including weighing,
 196 extraction, dilution, and injection) of the same endive sample injected within 12 h. Between-days
 197 reproducibility was defined as the RSD % for 54 technical replicates of a quality control sample obtained by
 198 mixing the powdered tissue of 10 chicons, during two months of analysis.

199 5.7 Data analysis

200 All statistical calculations were computed using Revolution R Open 3.2.1 (R Development Core Team, 2015)
201 linked with the BLAS/LAPCK libraries. The *lm()* and *aov()* functions from the *base* package and the
202 *TukeyHSD()* function from the *stats* package were used for linear models, analysis of variance (ANOVA)
203 and Tukey's honest significant difference (HSD) tests respectively. The *ggplot2* package was used for data
204 plotting (Wickham, 2009). In the boxplots, drawn according to Tukey, the notch indicates the median, the limits
205 of the big box the interquartile range, the whiskers extend to the most extreme data point which is no more
206 than 1.5 times the interquartile range away from the box, and samples outside the range are shown as
207 individual points. The data for the experimental samples was corrected for recovery dividing by the recovery
208 factor. Data for the food tables was the same as listed in supporting materials table S1.

209 6 Results and discussion

210 6.1 Screening of the polar soluble fraction with HPLC-MS

211 The goal of this part of the work was to evaluate how many known analytes could be detected in HPLC-HILIC,
212 investigate the presence of unknowns, and if possible identify them. Therefore, mass spectrometry was chosen
213 as the detection method because of its ability to provide positive identification of analytes. Despite the
214 selectivity and sensitivity of the MS detector, it is still necessary to test a variety of stationary phase/eluent
215 combinations in order to minimize biases due to chromatographic selectivity and/or poor ionization efficiency.
216 In this work, four stationary phases and nine buffers, chosen to maximize the coverage of the chromatographic
217 selectivity space (Kumar, Heaton, & McCalley, 2013; Periat, Debrus, Rudaz, & Guillardme, 2013), were tested in
218 all possible combinations within the operating limits of the system. At the same time ionization mode, column
219 diameter, particle size and solvents were kept as constant as possible, in order to simplify the comparisons. The
220 performance of each individual column/eluent combination was evaluated for the number of analytes
221 detected, peak resolution, and signal to noise ratio with the injection of endive extract.

222 The two zwitterionic columns were characterized by poor performance (table 1). Even after repeated rinsing
223 with clean eluents, the Nucleodur HILIC column had such a high bleed that the MS inlet clogged rapidly bringing
224 down the signal-to-noise ratio. The ZIC-HILIC column suffered less from the bleed, but exhibited physical
225 failure of the packing bed under conditions within the manufacturer's limits. Background spectra from these
226 two columns are shown in the supporting materials, figures S1 and S2. The remaining two columns showed a
227 bleed within the limits commonly observed during operation of the LCMS and adequate packing robustness,
228 and were used for the subsequent analyses.

229 A total of 22 analytes were identified, ten of which were identified by means of commercially available
230 authentic standards, three were identified from the injection of diluted Belgian endive latex (Sessa et al.,
231 2000), seven were identified exclusively on the basis of their spectra from the Metlin library, and one was
232 identified based on literature (Abu-Reidah et al., 2013). The full list of analytes is given in table 2.

233 Sugars, glutamic, quinic and malic acid were expected to be found because they were either described as
234 abundant in nutrition tables or known as ubiquitous in the plant world. Reducing sugars gave a strong signal,
235 but suffered from poor peak shape at pH's ranging from neutral to acidic because of low rates of mutarotation.
236 Malic acid gave a surprisingly low signal. It is known that polycarboxylic acids and phosphates can be adsorbed
237 on the many stainless steel surfaces of an HPLC/MS (Myint, Uehara, Aoshima, & Oda, 2009; Pesek, Matyska, &
238 Fischer, 2011). Active sites were chelated with the use of EDTA as described in the materials and methods.
239 Injection of 100 mg/L of citric and malic acid standards showed that buffer choice was of the utmost
240 importance to the detection of these compounds. Acidic buffers gave blurred peaks or no signal at all, while
241 alkaline buffers gave satisfactory peak shape. It was concluded that such compounds were present at a low
242 level and adsorption losses were negligible. Glutamine and pyroglutamic acid are not listed in nutrition tables.
243 Their presence. However, is hardly surprising as they are sometimes measured together with glutamic acid in
244 older methods with poor selectivity (Shih, 1985). One further peak was identified as pyroglutamyl-hexose
245 based on accurate mass and retention time (Abu-Reidah et al., 2013). The major components of the endive

latex are lactucin-, deoxylactucin- and lactucopicrin oxalate are normally analyzed in reverse-phase mode (Sessa et al., 2000). Their structure, however, contains polar functional groups and therefore enables a weak retention in HILIC mode as well. Injection of inulin standards generated a series of regularly spaced peaks (representing fructans of increasing chain lengths) not present in the Belgian endive extract.

The best separation was obtained with the combination of the Xbridge amide column and ammonium bicarbonate buffer (see figure 1), for which a run made in ESI positive mode was carried out as well. In this condition, all the analytes detectable with other buffers and columns were present, plus some unique ones. It must be highlighted that the Luna diol and the Xbridge amide performed similarly given similar chromatographic conditions, but the former is not amenable to use with alkaline buffers. The bicarbonate buffer gave a sugar mutarotation and signal intensity for neutral compounds as good as the ammonia, but at the same time showed a much better peak shape for charged compounds. The chelant properties of the bicarbonate anion allowed elimination of EDTA from the sample. Detection of alkaline metals peaks was then possible under ESI, as clusters of potassium and sodium hydrogen carbonate of the formula $[\text{CO}_3]^{2-} + n[\text{HCO}_3]^- + (n+1)[\text{K or Na}]^+$. Full spectra are shown in supporting materials, picture S3.

6.2 Method transfer to ELSD

Having determined the analytes amenable to HILIC, the next task was to transfer the method from MS to ELSD. The focus on this application was put on quantitative analysis, speed and reproducibility in the long term, sacrificing detection of the least abundant molecules. Initial tests under the same conditions as described in the previous section could detect only five of the analytes found with the MS (fructose, glucose, sucrose, glutamine and potassium), and a seventh analyte not detectable in MS (chloride). Quinic acid and malic acid, which were the largest peaks after the sugars in MS, could not be detected in ELSD. Injection of authentic standards generated detectable peaks, confirming that the system was able to elute and separate these analytes but their concentration was below detection limit.

270 Considering that alkaline buffers were not required due to the low amount of organic acids, high temperatures
271 were considered to achieve fast mutarotation. In turn, high temperatures lowered the viscosity of the solvent,
272 allowing use of a shorter column with a smaller particle size and thus shorter analysis times with the same
273 resolution. It was calculated that using an eluent composed of 75/25 acetonitrile/water with a 3 x 100 mm x
274 1.7 μm column at 70 °C, keeping the same linear velocity as the HPLC-MS, would generate a backpressure of
275 200 bars and peak widths of 6 s. This is well within the limits of traditional HPLC instrumentation.

276 The optimal chromatographic conditions were found to be as follows: ACN/water 75:25, ammonium acetate 3
277 mM, 80 °C, flow 0.6 mL·min⁻¹. A 1/20 dilution of the endive extract, obtained by pipetting 50 μL of extract into
278 950 μL of mobile phase, gave an optimal compromise between sensitivity and risk of overloading. There was no
279 attempt to optimize the extraction process as the sample extraction protocol had already been optimized for
280 the determination of the sesquiterpene lactones on the same samples. Different sugars were tested as
281 standards, with palatinose being the only one giving a satisfactory peak shape and resolution not co-eluting
282 with any of the analytes (figure 2). The finalized protocol is described in the supporting materials.

283 A log/log calibration was chosen because it is the best approximation of the response of an ELSD detector as
284 predicted by theory (Oppenheimer & Mourey, 1985). Linearity, limits of detection, recoveries and
285 reproducibility (shown in table 3) were found to be satisfying for the task at hand.

286 6.3 A practical application case

287 The optimized protocol was used to determine the content of polar analytes in 210 chicon samples, to show
288 the applicability of the protocol to real-world samples. Samples came from three different varieties of Belgian
289 endive, forced under the same conditions. The finalized protocol is fully described in materials and methods.
290 The range of the recovery-corrected concentrations for the varieties analyzed are reported in figure 3, together
291 with the values obtained from the nutrition tables. The values measured are in the same order of magnitude as
292 those listed in the most complete nutritional table, the Austrian food nutrition tables (Denkwerkzeuge &

293 Bernd, 2014). Our measurements indicate a slightly lower concentration of chloride and a markedly lower
294 concentration of fructose and sucrose. When looking at the sum of the simple sugars, values are in line with
295 the tables, and slightly higher when considering the sum of all analytes of interest.

296 An ANOVA followed by a Tukey's HSD test confirmed that three analytes were present in markedly different
297 levels between varieties, with p-values lower than 0.001. Notably potassium was present in higher level in
298 Symphonie, followed by Vintor, and then Takine. Fructose was much more abundant in Takine, while being
299 present in similar amounts in the other two. Regarding glutamine, Vintor and Symphonie had similar levels,
300 lower than Takine. This data seems to support anecdotal evidence from the farmers that Takine is sweeter than
301 other common varieties and hybrids.

302 7 Conclusions

303 For the first time, HILIC has been successfully applied to analyze the polar fraction of Belgian endive satisfying
304 the goals initially set. When comparing this method to those described in literature, many pitfalls are evident.
305 Methods targeted towards an individual class of analytes, or using more sensitive detectors, achieve better
306 accuracies and sensitivities several orders of magnitude larger. However, our method has three key
307 advantages: ruggedness, cheapness, and flexibility, which originate from the combination of sample
308 preparation, column choice and hardware requirements. Regarding ruggedness, minimalistic preparation of
309 samples and eluents combined with the characteristics of the columns deliver a constant performance. Peak
310 shapes and retention times were stable even after 2000 injections. Regarding cheapness, sample preparation
311 and ability to run on very common hardware contribute towards a low cost per sample. Regarding flexibility,
312 the Xbridge column is available in a wide choices of formats, and selectivity could be easily tuned with small
313 changes to the mobile phase. This allows scaling towards greater speed or resolution as required by different
314 matrixes.

315 These characteristics indicate the method might be readily adopted by the food industry. Two important
316 potential applications could be quality control of vegetable juices (as end product or semi-finished ingredient)
317 and batch grading and sorting of fresh produce. Sugars and salts are their main soluble components influencing
318 their sensory and technological qualities, and ultimately determining their economic value on the market. As
319 such, the method is currently being used in our lab to routinely screen Belgian endive samples.

320 8 Acknowledgments

321 The authors thank the Sofie Mannaerts for the technical work, Inagro vzw for the procurement of the
322 materials, and Waters Belgium for the gift of the mass spectrometer. The research was funded by the Flemish
323 agency for Innovation by Science and Technology (IWT project 090930).

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431

Table1

Modifier	Column	Nucleodur	ZIC-HILIC	Luna diol	Xbridge amide
	HILIC				
Ammonium acetate pH 4.5		Off scale	9.8	3.2	2.1
Ammonium acetate pH 6.8		Off scale	9.3	1.5	1.0
Formic acid 0.1 %		Off scale	1.0	0.8 *	1.2 *
Ammonium formate pH 3.5				0.5 *	0.3 *
Ammonium formate pH 6.5		Off scale	5.6	1.8 *	0.4 *
Ammonium formate pH 9.5					0.2 *
Ammonium bicarbonate pH 7.7					0.5 *
Triethylamine 0.1%					3.7
Ammonia 0.1%					0.7 *

Table 1: TIC background signal (arbitrary units, x 1000) of different columns/eluent system combinations, measured at the start of a blank run. Cells of Nucleodur HILIC column are listed as “off scale” because bleeding obstructed the detector inlet, preventing reliable quantification of signal strength. Cells in bold marked with an asterisk denote conditions chosen for further analyses.

table 2

peak N.	compound	identification	ESI -			ESI +		
			measured mass	calculated mass	fragments	measured mass	calculated mass	fragments
1	lactucopicrin oxalate	Standard	481.1178	481.1140	-	483.1276	483.1291	-
2	deoxylactucin oxalate	Standard	331.0826	331.2975	-	333.0951	333.0974	-
3	lactucin oxalate	Standard	347.076	347.0767	-	349.0837	349.0923	-
4	pyroglutamic acid	Metlin	128.0347	128.0353	out of instrument range	130.0519	130.0499	too weak
5	phenylalanine	Standard	164.0737	164.0717	147.0420	166.0816	166.0863	120.0792
6	quinic acid	Standard	191.0580	191.0561	out of instrument range	-	-	-
7	isoleucine	Metlin	130.0875	130.0874	out of instrument range	132.0971	132.1019	out of instrument range
8	leucine	standard	130.0869	130.0874	out of instrument range	132.0975	132.1019	out of instrument range
9	pyroglutamyl-hexose	Abu-Reidah 2013	290.0878	290.0881	too weak	-	-	
10	fructose	Standard	179.0558	179.0561	161.0467;	-	-	-
11	glucose	Standard	179.0563	179.0561	143.0362; 113.0251 161.0446;	-	-	-
12	malic acid	Standard	133.0118	133.0142	119.026; 113.0253	-	-	-
13	glutamic acid	Metlin	146.0463	146.0459	115.0029	-	-	-
14	glutamyl-hexose	Abu-Reidah 2013	307.1143	307.1147	128.0364; 102.0537	309.1115	309.1300	291.1072; 273.1019
15	asparagine	Metlin	131.0473	131.0462	217.0831; 145.0631	-	-	-
16	glutamine	Standard	145.0605	145.0619	113.0354; 114.0237	-	-	-
					127.0525; 128.0426	-	-	-

17	sucrose	Standard	341.1051	341.1089	179.0577	-	-	-
18	UDP monosaccharide	Metlin	565.0546	565.0477	too weak	-	-	-
19	tetrasaccharide	Metlin	665.2250	665.2146	too weak	-	-	-
20	potassium	Standard	clusters	-	-	clusters	-	-
21	sodium	Standard	clusters	-	-	clusters	-	-
22	arginine	Metlin	173.0968	173.1044	131.0833	175.1151	175.1190	-

Table 2: analytes identified on the Xbridge amide column run with ammonium bicarbonate buffer. The “identification” column reads as follows: standard – injection of authentic standard; Metlin: matching of accurate mass and fragments with the Metlin database; Abu-Reidah 2013 – matching of accurate mass and fragments with the analytes described in the aforementioned paper

table 3

Analyte	calibration range (g·Kg ⁻¹ fresh weight)	calibration R ²	spike recovery %	limit of detection (g·Kg ⁻¹ fresh weight)	Within day RSD %	Between days RSD %
fructose	0.781 - 100	0.996	90.526	NA	1.5240	3.525
glucose	0.781 - 100	0.998	94.323	NA	3.096	2.405
sucrose	0.625 - 10	0.999	79.946	NA	6.774	4.678
glutamine	0- 10	0.981	108.423	0.183	5.844	17.929
potassium	0 – 52.7	0.989	109.656	0.282	18.560	12.713
chloride	0 – 47.2	0.999	80.282	0.301	9.626	9.552

Table 3: details of the quantitative calibration in ELSD. Limits of detection were not calculated for the tree sugars because they were always present at abundant levels.

Figure 1

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Figure 2

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Figure 3
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Figure captions

Figure 1: HPLC-MS negative mode chromatogram of Belgian endive extract. Conditions: 10 mM ammonium bicarbonate buffer on the Xbridge column, no EDTA added. Black line: TIC, colored lines: XIC of identified analytes. Peak identification and numbering as in table 2.

Figure 2: ELSD chromatograms of internal standards and endive extract in the optimized conditions. Details: Xbridge amide column (3 x 100 mm x 1.7 μ m), ACN/water 75:25, ammonium acetate 3 mM, 80 °C, flow 0.6 mL·min⁻¹.

Figure 3: Analytes concentration in different varieties. Each subplot reports data for an analyte. Individual boxes represent the distribution of values for (left to right): recovery corrected experimental data for varieties Takine, Vintor, and Symphonie; and nutrition tables. Brackets over the boxes indicate the adjusted p-values of the Tukey's HSD test. Calculations for the plot are described in "materials and methods", subsection "data analysis".