Isotachophoretic Determination of Glucosamine and Chondroitin Sulphate in Dietary Supplements

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Abstract


Glucosamine and chondroitin sulfate, components of normal cartilage, are used as ingredients in dietary supplements intended to treat osteoarthritis and/or to support joint health. Of concern is the documented lack of quality in many of the marketed products. We present here a capillary isotachophoretic method for the determination of glucosamine and chondroitin sulfate in dietary supplements. Cationic analysis of glucosamine was performed with a leading electrolyte consisting of 10mM NH₄OH + 20mM acetic acid. As the leading electrolyte for anionic analysis of chondroitin sulphate, a mixture of 5mM HCl + 10mM glycylglycine + 0.05% of 2-hydroxyethylcellulose was used. The solution of 10mM citric acid served as the terminating electrolyte for both glucosamine and chondroitin sulfate analyses. The analytes were detected by conductivity and UV detectors. The characteristics of the method, i.e., linearity, accuracy, repeatability, and quantitation limit, were evaluated. On a set of 35 samples of marketed dietary supplements did we prove that the capillary isotachophoresis is a suitable method for the routine analysis of glucosamine and chondroitin sulfate.

Keywords: capillary isotachophoresis; nutraceuticals; food analysis

Osteoarthritis is the most common degenerative joint disease of the middle-aged and elderly people; the patients affected by this disease suffer from pain and the loss of function. Glucosamine (GA) and chondroitin sulfate (CS) are components of normal cartilage. They are used as ingredients (together or separately) in dietary supplements intended to support the joint health. Due to the increased prevalence of osteoarthritis and the lack of effective therapies, interest is given to the use of these dietary supplements as therapeutic agents for osteoarthritis. Usual daily dose of glucosamine sulphate and chondroitin sulphate is 1500 mg and 1200 mg, respectively (Messier et al. 2007; Bruyere et al. 2008; Jackson et al. 2010).

There are plenty of such dietary supplements on the marketplace. Of concern is the documented lack of quality in many of the marketed products both in the finished dosage forms and raw materials (Adebowale et al. 2000; Barnhill et al. 2006).

Glucosamine (2-amino-2-deoxy-\text{d-glucose}) is the principal component of glycosaminoglycans that form the matrix of connective tissues in the human body. Glucosamine, also known as chitosamine, is the basic monomer of chitin. Chitin is a biopolymer composed of \text{N}-acetyl-d-glucosamine, which can be found in exoskeletons of invertebrate marine creatures such as oysters, crabs, or shrimps. Glucosamine is usually prepared from chitin by hydrolysis using strong mineral acid (sulphuric or hydrochloric) (Mojarrad et al. 2007). During this reaction, chitin is deacetylated and depolymerised to glucosamine salt of the acid used (Figures 1a–c). The obtained high quality glucosamine hydrochloride is stable, while glucosamine sulfate is not. It is very hygroscopic.
and degrades rapidly when exposed to moisture. To overcome this disadvantage, glucosamine sulphate is made from glucosamine hydrochloride by co-crystallisation with potassium or sodium sulphate to yield glucosamine sulphate 2 KCl or 2 NaCl. The content of the glucosamine base (active substance) in glucosamine hydrochloride is 83% (w/w) while in glucosamine sulphate 2 KCl it is only 59% (w/w). Most of the dietary supplements contain glucosamine sulphate 2 KCl rather than glucosamine hydrochloride.

Glucosamine in pharmaceutical formulations, nutraceuticals, and raw bulk materials is determined by HPLC with RI detection (El-Saharty & Bary 2002), evaporative light scattering detection (JACYN & THRALL 2004), direct UV detection at 195 nm (Shao et al. 2004), and UV detection at 254 nm after derivatisation with phenylisothiocyanate (Liang et al. 1999). Besides HPLC technique, capillary electrophoresis (CE) with UV detection (214 nm) after the derivatisation of GA with anthranilic acid (Qi et al. 2006) or with conductometric detection (JAC et al. 2008) without derivatisation have been used. Other described techniques suitable for the analysis of GA involve spectrophotometry after derivatisation with ninhydrin (Wu et al. 2005), and high performance thin-layer chromatography (ESTERS et al. 2006).

Chondroitin sulphates (Figures 1d–f) are naturally occurring glycosaminoglycans containing disaccharide repeating units formed by hexuronic acid and hexosamine residue. The eminent feature of the molecular structure of CS is the presence of a sulphate ester group in the hexosamine and/or hexuronic acid residue, which gives the CS molecule a highly negative ion charge. CS as a part of proteoglycans is present on the cell surfaces and in the extracellular matrix of almost all animal tissues. It is considered as an important part of the composition of the joint cartilage. Chondroitin sulphate is present in a number of forms; the most common forms are chondroitin sulphate A (Figure 1d), chondroitin sulphate B also known as dermatan sulphate (Figure 1e), and chondroitin sulphate C (Figure 1f). The molecular weight of CS varies between 9000 Da and 93 000 Da (Sim et al. 2005). CS is isolated mainly from bovine or porcine cartilage (source of CS-A) and from shark cartilage.
cartilage as a source of CS-C. The isolation process involves extraction using alkaline solutions or enzymes, alcoholic precipitation, recovery of solids by centrifugation and purification (Schiardi et al. 2010). Fish derived CS is currently referred to as the best quality raw material on the market, considering the degree of purity and sulphation pattern. The identity and quality of CS is the major issue related to its therapeutic activity. Some dietary supplements contain less than the labelled amount of CS, in some cases as little as 10%. Due to the poor CS quality in some nutraceuticals, there is a need for stricter regulations regarding the quality of CS and specific and

<table>
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<th>Sample</th>
<th>Form of DS</th>
<th>Active components</th>
<th>Daily dose</th>
<th>form of DS</th>
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<th>GA&lt;sup&gt;1&lt;/sup&gt; (mg)</th>
<th>MSM (mg)</th>
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<td>1200</td>
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CS – chondroitin sulfate; GA – glucosamine; MSM – methylsulfonylmethane; HA – hyaluronic acid; HC – hydrolysed collagen; GA*HCl – glucosamine hydrochloride; GA*SO<sub>4</sub> – glucosamine sulphate; GA<sup>1</sup> – expressed as glucosamine hydrochloride

CS – chondroitin sulfate; GA – glucosamine; MSM – methylsulfonylmethane; HA – hyaluronic acid; HC – hydrolysed collagen; GA*HCl – glucosamine hydrochloride; GA*SO<sub>4</sub> – glucosamine sulphate; GA<sup>1</sup> – expressed as glucosamine hydrochloride
accurate analytical procedures to confirm the purity
and label claims for both the raw bulk materials
and finished products (Volpi 2009).

The official method mentioned in European
Pharmacopoeia concerning the analysis of chon-
droitin sulphate as a bulk material or as an active
principle in pharmaceutical preparations is based
on the infrared spectroscopy and electrophoretic
identification, and on a photometric titration
assay using cetylpyridium chloride (European
Pharmacopoeia 2006). The most common spectro-
photometric method for the determination of CS
in pharmaceuticals and/or nutraceuticals is based
on the chromogenic reaction between carbazole
and hexuronic acid produced by acid hydrolysis of
CS (Bitter & Muir 1962). It is noteworthy that
both the titration and the spectrophotometrical
methods can be influenced by the sample matrix
of dietary supplements (collagen, hyaluronic acids,
etc.) giving usually positively false results. Other,
more selective methods, involving size-exclusion
chromatography (Toida et al. 1997; Choi et al.
2003), ion-pair HPLC (Jin et al. 2009), and CE
(Malavaki et al. 2008) for the determination of chondroitin sulphate in pharmaceutical and/or
nutraceutical preparations have been reported.
Highly selective methods based on enzymic diges-
tion of CS using chondroitinase followed by HPLC
(Grøndhal et al. 2011) or CE (Karamanos et
al. 1995; Okamota et al. 2004; Malavaki et al.
2008) determination of chondroitin disaccharides
have been also published.

Capillary isotachophoresis (cITP) equipped with
a conductivity detector offers an alternative to the
existing methods for the determination of GA and
CS in dietary supplements. As far as we know from
the literature, the application of cITP has not been
published up to know. That is why we present here
a capillary isotachophoretic method for the deter-
mination of GA and CS in dietary supplements.

MATERIAL AND METHODS

Chemicals and samples. The standards of
glucosamine hydrochloride, chondroitin sul-
phate A, chondroitin sulphate B, chondroitin
sulphate C, chondroitinase ABC, unsaturated
chondroitin disaccharides 2-acetamido-2-deoxy-3-
O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic
acid)-4-O-sulpho-d-galactose (Δdi-mono4S), and
2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-
hex-4-enopyranosyluronic acid)-6-O-sulpho-d-ga-
lactose (Δdi-mono6S) were purchased from Sigma-
Aldrich (Prague, Czech Republic). The chemicals
for the electrolyte preparation, i.e., hydrochloric
acid volumetric standard (1 mol/l solution in wa-
ter), acetic acid volumetric standard (1 mol/l solu-
tion in water), ammonium hydroxide volumetric
standard (5 mol/l solution in water), glycylglycine
(GLYGLY), and 2-hydroxyethylcellulose (HEC)
were purchased also from Sigma-Aldrich (Prague,
Czech Republic). Citric acid was obtained from
Lach-Ner, Ltd. (Neratovice, Czech Republic). All
chemicals were of analytical grade. Deionised water
of Milli-Q quality (electrical resistivity 18.2 MΩcm)
was used for the electrolyte, standard, and sample
preparations. The samples of dietary supplements
(Table 1) were obtained from the dietary supple-
ment manufacturers and/or from the domestic
market.

Instrumentation. Isotachophoretic analyses were
carried with the help of manual column coupling
electrophoretic analyser EA 101 with contact con-

Table 2. Conditions of ITP analysis of glucosamine and chondroitin sulphate

<table>
<thead>
<tr>
<th></th>
<th>Cationic analysis of glucosamine</th>
<th>Anionic analysis of chondroitin sulphate</th>
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<tr>
<td>Leading electrolyte</td>
<td>10mM NH₄OH + 20mM acetic acid</td>
<td>5mM HCl + 10mM GLYGLY + 0.05% HEC</td>
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<tr>
<td>Terminating electrolyte</td>
<td>10mM citric acid</td>
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<td>Driving current (µA)</td>
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<td>Analytical capillary</td>
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<td>Sample load (µl)*</td>
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<tr>
<td>Detection</td>
<td>conductivity and UV at 254 nm</td>
<td>conductivity and UV at 254 nm</td>
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<tr>
<td>Analysis time</td>
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</table>

*sample valve with the fixed internal loop; GLYGLY – glycylglycine; HEC – 2-hydroxyethylcellulose
ductivity detectors and a UV detector operating at 254 nm (Villa-Labeco, Spisska Nova Ves, Slovak Republic). The separation compartment of EA 101 analyser consisted of the pre-separation FEP capillary (90 mm × 0.8 mm i.d.) coupled with the analytical FEP capillary (90 mm × 0.3 mm i.d.). EA 101 was controlled with the help of PC software package ITTPPro32 (KasComp Ltd., Bratislava, Slovak Republic) supplied with the analyser. The instrument operates with the hydrodynamically closed separation system (Kaniansky et al. 1997), i.e., electro-osmotic flow is suppressed.

**Conditions of analyses, standard and sample preparation.** GA and CS in dietary supplements were determined as an anion and a cation, respectively. The conditions of analysis are described in Table 2.

External standard technique (five-point calibration) was used for the quantitative analyses of CS and GA. The calibration solutions of CS (20, 40, 60, 80, and 100 mg/l) were prepared from the stock solution containing 1000 mg of CS/l of demineralised water. The calibration solutions of GA (5, 10, 20, 50, and 100 mg/l) were prepared from the stock solution (1 g/l GA in demineralised water). CS and GA were extracted from the dietary supplements by demineralised water. One tablet, capsule, or recommended dose of powdered or liquid samples was placed (or weighed) into 250-ml volumetric flask and 200 ml of demineralised water was added. The flask was put into an ultrasonic bath (wattage 300 W) for approximately 30 minutes. The extraction was finished when the total disintegration of the tablet was reached. The extract obtained was diluted and filtered (through filter paper) prior to analysis.

**Method validation.** The cITP method validation was performed according to the International Conference on Harmonization guideline (ICH 1995, 1996) including parameters such as linearity, precision, accuracy, limit of quantitation (LOQ), and limit of detection (LOD). The calibration curves were constructed from the step length vs. concentration of GA and/or CS. Linear regression was applied for the calculation of the intercept, slope, and correlation coefficients of GA and CS calibration curves.

The precision was assessed by the determination of GA and CS (six replicas) in two different samples (including extraction, dilution, and cITP analysis). The intra-day precision was expressed as the relative standard deviation (RSD in %) of the analyses of two samples performed within one day. The inter-day precision was expressed as combined RSD (%) of the analyses of two samples performed within two days.

The accuracy of the cITP method was assessed by the determination of GA and CS (three replicas) in a sample spiked with GA and/or CS. The accuracy was expressed as the average recovery (%) of the standard addition at two different levels (50 and 100% additions of the declared concentration of GA and/or CS). For the accuracy and precision measurements, 20 tablets of the sample were triturated in a grinding mortar and thoroughly homogenised. The sample amount corresponding to one tablet was used for the extraction. The spiked samples were prepared by weighing the homogenised sample plus an appropriate amount of GA and/or CS standard into the extraction flask.

The limit of detection (calculated from the calibration equation) corresponds to the concentration of GA and/or CS which gives the minimum detectable zone length equal to one second. The limit of quantitation is calculated as the treble of the detection limit.

**RESULTS AND DISCUSSION**

GA as a medium-strong base (pK₈ 8 ± 0.2) (Blas-kó et al. 1997; Jáč et al. 2008) is protonised (glucosaminium) up to pH 10 and can be determined as a cation within a wide pH range. The effect of pH of the leading electrolyte on the electrophoretic migration of GA was examined in the range 3.5–8 (data not shown). At a low pH (< 4.5), the potential excipients of the dietary supplement (mainly hydrolysed collagen) might interfere with GA. At pH > 6, the separation of GA from the potential interferers was excellent. However, due to the relatively low limiting electrophoretic mobility of GA (comparable to the mobility of tetrabutylammonium cation) problems occurred with selecting sufficiently slow terminating cation. Moreover, the start of the analysis was delayed because the low mobility terminator generates high voltage along the capillary and thus the driving current must be decreased. In view of the costs of both the separation and analysis, we selected 10 mM NH₄OH (or KOH) + 20 mM acetic acid (pH 4.7) as the optimal leading electrolyte and 10 mM citric acid as the terminating electrolyte (hydroxonium is used as terminator). The isotachopherograms of GA standard and the sample No. 33 are shown in Figure 2. It is evident from the conductivity records that GA is fully separated from the sample matrix.
The disaccharide unit of chondroitin contains one carboxylic group (pKₐ ~ 3.3 (Tommeraas & Wahlund 2009)) and one (or more) O-sulphate group (pKₐ ~ –1). CS is thus a strong poly-acid with a high charge density and is negatively charged even at a low pH (< 3). This property of CS allows performing an electrophoretic separation in acidic electrolytes. We studied the influence of different electrolyte systems in the pH range of 2.5–6 on the electrophoretic mobility of chondroitin sulphates (CS-A, CS-B, and CS-C) and on their separation from common excipients of dietary supplements (data not shown). We found out that the electrolyte system consisting of 5mM HCl + 10mM GLYGLY + 0.05% HEC (pH 3.2) as the leading electrolyte and 10mM citric acid as the terminating electrolyte gave the best results. Under these acidic conditions, the risk of interference of anionic matrix components with CS is minimised. For example, desulphated chondroitin (hyaluronate) as a possible impurity of CS has a lower mobility than the terminator (citrate) and thus does not disturb the analysis. Acesulfam K and a high content of hydrolysed collagen (> 10 g/100 g) were the only interferents that we met. Acesulfam K migrates very closely to CS, however, it can be easily detected in the UV trace due to its strong absorption at 254 nm. Hydrolysed collagen is positively charged at a low pH and is able to form a complex with chondroitin and to decrease its mobility resulting in the absence of CS step in the isotachopherogram. The problem with collagen can be solved by performing the analysis at a higher pH. For example, collagen is not highly protonised at pH 6 (very weak complex with chondroitin) and CS is separated and determined. However, at this pH a risk occurs of CS interference with other excipients or non-chondroitin polysaccharides.

The isotachophoreograms of the blank analysis and standards of CS-A, CS-B, and CS-C are depicted in Figures 3a–d. All the CS standars tested had almost identical mobilities (similar step heights on the conductivity record) due to similar charge densities (number of sulphate groups per disaccharide unit) and thus could not be separated by cITP method. The molecular weight of CS has a negligible influence on

Figure 2. Isotachopherograms of blank, glucosamine standard and sample; trace from the conductivity (black) and the UV (254 nm) detector (gray) of the analytical capillary, respectively: (a) isotachopherogram blank (demineralized water); (b) isotachopherogram of glucosamine standard (20 mg/l); (c) isotachopherogram of dietary supplement sample No 33 (1 tablet/250 ml, 50x diluted, ca 150 µmol GA/l).
the electrophoretic mobility of CS (MALAVAKI et al. 2008). It is clear from the UV trace (Figures 3b–d) that all Sigma CS standards contain “impurities” absorbing at 254 nm. UV spectrum of “impurities” has a broad maximum at 228–236 nm (data not shown). It led us to the presumption that the im-
purities could be unsaturated oligosaccharides (up to decasaccharides) as products of partial enzymic cleavage of chondroitin sulphate by chondroitinase formed within the isolation or purification of chondroitin sulphate (Zhang et al. 2009). Chondroitinase cleaves the 1β→4 glycosidic bonds between N-acetylgalactosamine and uronic acid of CS and releases unsaturated oligosaccharides strongly absorbing UV light. The final products of the cleavage are variously sulphated disaccharides depending on the original CS (Saito et al. 1968). CS-A and CS-B give Δdi-mono4S while CS-C gives Δdi-mono6S.

The isotachophoreogram of a mixture of CS-A, Δdi-mono4S, and Δdi-mono6S (Figure 3e) clearly shows that chondroitin is separated from these disaccharides. The hydrolysis product of CS-A is shown in Figure 3f. It is evident from this record that there is no step of chondroitin and/or “impurities”. It confirms our hypothesis that “impurities” are unsaturated oligosaccharides. Furthermore, from the record is clear that the standard of CS-A is a mixture of CS-C and CS-A. It contains both Δdi-mono4S and Δdi-mono6S. The skewed steps of disaccharides are due to the anomeric forms of hexosamines present at the reducing end of chondroitin disaccharides. These α- and β-anomers are not fully separated under the given conditions. In the isotachophoreogram another step (UV absorbing) is present with the electrophoretic mobility higher than that of the original CS (lower step height). This probably represents disaccharides with more than one sulphate groups in their molecules. Phosphate migrating between CS and disaccharides comes from chondroitinase ABC.

Validation of cITP determination of GA

The validation was carried out under optimal conditions (Table 2) using standard solution of GA (10–100 mg/l) and selected samples of dietary supplements (33 and 34). The values of the characteristics of cITP method are summarised in Table 3. The cITP method is characterised by excellent linearity ($r > 0.999$), good precision ($RSD = 1.82\%$), high accuracy (recovery 98.8 ± 1.7%), and high sensitivity (LOQ = 2.4 mg/l). We applied cITP for the analyses of dietary supplements samples. Some of them were obtained directly from a manufacturer; some were purchased on the domestic market. The results of analyses of 33 samples are summarised in Figure 4a. The GA content found was expressed as the percentage of the declared content of the labelled form of GA. Only 21 samples (64%) contained more than

Table 3. Method characteristics of isotachophoretic determination of GA and CS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>GA value</th>
<th>CS value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSH (–)</td>
<td>0.79 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Precision (RSD in %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day¹</td>
<td>1.82</td>
<td>1.53</td>
</tr>
<tr>
<td>Inter-day²</td>
<td>1.67</td>
<td>1.94</td>
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<tr>
<td>Accuracy (% recovery)³</td>
<td>98.8 ± 1.7</td>
<td>99.7 ± 2.6</td>
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<tr>
<td>Linearity (mg/l)</td>
<td>10–100</td>
<td>20–200</td>
</tr>
<tr>
<td>Intercept (mg/l)</td>
<td>0.2 ± 0.4</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>Slope (mg/l/s)</td>
<td>0.555 ± 0.005</td>
<td>1.34 ± 0.01</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>Limit of detection (mg/l)⁴</td>
<td>0.8</td>
<td>3</td>
</tr>
<tr>
<td>Limit of quantitation (mg/l)⁵</td>
<td>2.4</td>
<td>9</td>
</tr>
</tbody>
</table>

RSH – relative step height; it is average value from calibration analyses (for GA) and from the analyses of CS-A, CS-B and CS-C (for Cs);¹ analysis of sample No 33 and 34 within one day ($n = 6$); analysed concentration of GA was ca 20 mg/l and 30 mg/l and analysis of sample No 16 and No 35 within one day ($n = 6$); analysed concentration of CS was 80 and 60 mg/l;² analysis of sample No 33 and 34 for GA and sample No. 16 and No. 35 for CA within two days ($n = 12$);³ analysis of sample No 33; analysed concentration of GA was ca 20 mg/l; 50 and 100% standard addition, i.e., 10 and 20 mg/l and analysis of sample 16; analysed concentration of CS was 40 mg/l; 50 and 100% standard addition (CS-A), i.e., 20 and 40 mg/l;⁴ calculated as a minimal detectable step length (1 s);⁵ calculated as the treble of the limit of detection.
90% of the declared amount of GA. In our opinion, the incorrectly labelled form of GA is the main reason for such findings. Only a few samples were labelled with the proper form of GA, e.g., glucosamine hydrochloride or glucosamine sulphate*2 KCl. Most of the samples stated to contain glucosamine sulphate, but actually glucosamine sulphate*2 KCl had been used. As we mentioned before, glucosamine sulphate is an unstable substance and that is why this form is not used. The differences in GA content are great because of great differences in molecular weight (Figure 1). If we recalculated the content of GA as (GA)*2 sulphate*2 KCl, more than 90% of the samples would be satisfactory. It is noteworthy that in such case the customer buys mineral salts at the price of glucosamine.

Validation of cITP determination of CS

The characteristics of the method of CS determination using cITP were evaluated in the same way as in the case of GA. The calculated parameters are based on the results of the analyses carried out under optimal conditions (Table 2) using standard solution of CS-A (20–200 mg/l) and selected samples of dietary supplements (16 and 35). The results obtained are summarised in Table 3. The method is characterised by excellent linearity ($r > 0.999$), good precision ($RSD = 1.94\%$), high accuracy (recovery $99.7 \pm 2.6\%$), and high sensitivity (LOQ = 9 mg/l). The results of the analyses of 22 samples are summarised in Figure 4b. The CS content found was expressed as the percentage of

Figure 4. Results of ITP analyses of food supplements sample; found concentration of GA (a) and CS (b) was expressed as a percentage of the declared content.
the labelled content of CS. Only 6 samples (27%) contained more than 90% of the declared amount of CS. Chondroitin raw material of a low quality is the main reason for such results.

CONCLUSIONS

We presented cITP method for the determination of glucosamine and chondroitin in raw materials and dietary supplements such as tablets, capsules or liquid formulations. The developed methods were validated according to ICH guideline. The presented results show that cITP method fulfils general requirements for analytical methods and is thus suitable for the analysis of glucosamine and chondroitin in both raw material and dietary supplements. Low laboriousness (water extraction and filtration only) and low running costs are the important features of cITP methods especially for routine analyses. The cITP can be an expedient alternative to HPLC or CZE.

References


Received for publication March 6, 2012
Accepted after corrections June 26, 2012